1968

Parathyroid Extract, Thyrocalcitonin and the Metabolism of Bone in Vitro

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PARATHYROID EXTRACT, THYROCALCITONIN AND THE METABOLISM OF BONE IN VITRO

by

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June

1968

LIFE

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Mr. DeLong is the co-author of the following papers. "Parathyroid Extract-Induced Alterations in the Oxidation of Organic Acids in Bone".

A.F. DeLong and M.V. L'Heureux, Federation Proceedings 26 811, 1967.

"The Influence of Parathyroid Extract on the Oxidation of Organic Acids in Bone".

Allyn F. DeLong and Maurice V. L'Heureux, Life Sciences 6 2161 - 2169, 1967.

"Parathyroid Extract, Calcium Ion Concentration and the Metabolism of Pyruvate-2-^{14}C by Bone Homogenate".

A.F. DeLong and M.V. L'Heureux, Federation Proceedings 27 690, 1968.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Maurice V. L'Heureux for his guidance and constructive criticism during the preparation of this dissertation. The author also wishes to express his acknowledgements to the other staff members and fellow graduate students who have contributed to his work by exchange of ideas and whose personalities have made graduate education even more interesting.

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TABLE OF ABBREVIATIONS

EDTA - Ethylenediaminetetraacetic Acid

NADP - Nicotinamide Adenine Dinucleotide Phosphate

PTE - Parathyroid Extract

PTH - Parathyroid Hormone

PTX - Parathyroidectomized

TCT - Thyrocalcitonin

TPTX - Thyroparathyroidectomized

CHAPTER I
INTRODUCTION

PARATHYROID HORMONE

History and Origin

Surgeons in the early nineteenth century noted that a common after-effect of the removal of the thyroid was the development of a neuromuscular syndrome ranging from mild muscle spasms to severe tetany. Approximately 50 years later the discrete entities which were called the parathyroid glands and believed to be non-functional, were found closely associated with the thyroid gland. In 1880 Sandström (102) first discovered the external parathyroid glands and in 1891 their existence was confirmed by Gley (46). The discovery of the internal parathyroid glands was demonstrated by Kohn in 1895 (64). Vassale and Generali (115) established the relationship between the parathyroid glands and tetany. Thyroidectomy performed without cognizance of the parathyroid glands resulted in tetany attributed to the removal of the thyroid gland. In 1908 MacCallum and Voegtlin (74) established the relationship between the nervous manifestations of parathyroidectomy and the level of serum calcium. Skeletal changes incident to hyperparathyroidism, although imperfectly understood, were first described by Von Recklinghausen (116).

During 1925 the parathyroid gland attained the status of an endocrine organ when Hanson (49) and Collip (28) independently accomplished the isolation of a physiologically active extract

from the glands. Thus was ushered in the modern era of parathyroid hormone research in which the chemistry and mode of action of parathyroid hormone have been intensely studied.

The site of secretion and storage of parathyroid has been linked to the chief cells of the parathyroid gland. Using the technique of immunofluorescence, Hargis et al. (50) demonstrated parathyroid hormone overlying the cytoplasm of the chief cells of bovine, human, and rat parathyroid glands. This technique showed no specific staining with oxyphil cells in human tissue, a cell type previously linked to parathyroid hormone secretion and storage.

The secretion of the hypercalcemic agent, parathyroid hormone, is governed directly by the concentration of calcium ion in blood (22, 30, 50, 86, 104). This relationship has been conclusively shown by measuring plasma hormone concentrations following changes in glandular plasma calcium concentration effected by perfusion. Care et al. (22) using radioimmunological assay techniques demonstrated that perfusion of goat parathyroid gland with blood containing 14% calcium led to an 80% decrease in parathyroid hormone secretion within one-half hour. Subsequent perfusion with low-calcium blood led to a rapid increase in hormone secretion to seven times the suppressed value. Recently, Ramberg et al. (93) have presented data to indicate that parathyroid hormone is continuously secreted at a rate which is proportional to plasma calcium concentration.

Assay

Most of the current assay procedures employ a modification of the Munson technique (81) which utilizes the parathyroid hormone-induced mobilization of calcium into the blood of rats as the parameter for assay. The use of thyroparathyroidectomized animals has greatly improved the index of precision of this assay due to the lack of interference from thyrocalcitonin secretion which might be expected to supress increases in plasma calcium following the administration of parathyroid hormone. The assay is sensitive to 2 USP units of parathyroid extract.

The concentration of parathyroid hormone in plasma can be measured by a much more sensitive technique developed by Berson et al. (14). The assay employs the principle of competitive inhibition of binding of highly purified parathyroid hormone, labeled with ^{131}I, to specific hormone antibodies which are produced in guinea pig. Extracts of the serum or tissue to be assayed displaces the antibody bound ^{131}I-labeled bovine parathyroid hormone according to the respective concentration of parathyroid hormone in the test material. Such an assay can detect as little as 10 uug of parathyroid hormone.

Chemistry

Nearly all studies concerning the isolation and characterization of parathyroid hormone have been confined to that entity extracted from bovine glands (7, 52, 73, 90, 97). Recent techniques employ a three stage isolation procedure: 1) acetone

dehydration and chloroform defatting of freshly frozen parathyroid glands, 2) extraction of the active principle from these acetone powders with either 90% phenol or a mixture of 8 M urea - 0.2 N HCl - 0.1 M cysteine followed by solvent and salt fractionation, 3) successive gel filtrations of the crude TCA precipitate using various grades of Sephadex and finally carboxymethylcellulose.

The hormone molecule obtained by either phenol or the urea - HCl - cysteine method is biologically and chemically the same (52). Potts and Aurbach (90) have reported the isolation of a minor component in the carboxymethylcellulose material which is biologically, immunochemically, and chemically similar to the major component but has a slightly different amino composition. These workers have presented the only detailed reports concerning the primary amino acid sequence of parathyroid hormone. They have isolated and determined the sequence of a carboxyterminal peptide consisting of 20 amino acid residues which retains most of the immunological activity and 30% of the biological activity of the parent hormone.

The loss of biological activity of parathyroid hormone by treatment with H_2O_2 or performic acid has been closely correlated with the rate of oxidation of methionine residues to methionine sulfoxide or methionine sulfone (95). Of the two methionine residues present in parathyroid hormone, only one is believed to be necessary for biological activity (90, 107).

Biological Response and Mode of Action

It has been clearly shown that parathyroid hormone has a direct action on both bone and kidney (6, 89) and to some extent on the gastrointestinal tract. The major function of the hormone is to maintain the calcium ion activity of the extracellular fluids within physiological limits. The total effect of the hormone on all three major target organs is to conserve body stores of calcium.

Rasmussen (94) presented one of the first detailed reports linking parathyroid hormone and the absorption of calcium from the gastrointestinal tract. He found that parathyroidectomy consistently decreased the absorption of calcium from the gut of experimental animals. It was concluded that parathyroid hormone is necessary for normal calcium absorption. Birge, Peck and Berman (16) reported that calcium absorption is increased in humans with hyperparathyroidism.

However, in order for parathyroid hormone to act on the absorption of calcium from gastrointestinal tract, vitamin D must be present in sufficient quantity (32). The administration of parathyroid hormone has no effect in stimulating the absorption of calcium from the gut of vitamin D-deficient animals (55). Conversly thyroparathyroidectomy of vitamin D-deficient animals does not decrease the absorption of calcium from the gut as it would in normal animals. It appears that parathyroid hormone action and vitamin D are intimately associated with respect to

calcium absorption.

The administration of parathyroid hormone to mammals produces a rapid increase in urinary excretion of phosphate and an initial decreased excretion of calcium and magnesium. Beuter et al. (15) showed that a reduced urinary excretion of phosphate followed parathyroidectomy. They also demonstrated that injection of parathyroid extract produced a hyperphosphaturia. Ito et al. (59) found a 70% increase in the tubular reabsorption of phosphate following parathyroidectomy.

There is evidence to indicate that changes in glomerular filtration rate are not responsible for hormonally-induced changes in ion excretion and that the renal tubule is the site at which the hormone acts (55, 77). Although the exact site of parathyroid action upon the renal tubule remains unsettled, most investigators favor the view that its major action is the inhibition of proximal tubular reabsorption of phosphate (23, 54, 77). Others have indicated that the action of parathyroid hormone may involve active phosphate secretion in the distal tubules. The studies of Nicholson (83) and Nicholson and Shepherd (84) on the nephron of the dog kidney which was selectively damaged by nephrotoxic agents suggested hormone stimulation of phosphate secretion. In either case the postulated mechanism of hormone action would be to increase the concentration of inorganic phosphate within renal tubular cells. In the proximal tubule, this would lead to diminished tubular reabsorption by

increasing the concentration gradient to be surmounted at the luminal border (84), and at the distal site where luminal phosphate concentration is lower it could lead to phosphate secretion.

Action in Bone

Since the skeletal system contains 98% of the body stores of calcium, parathyroid hormone regulation of plasma calcium must in some way involve bone. Parathyroid hormone has two major direct effects on bone: to promote resorption and to inhibit collagen synthesis in the formation of bone. Gaillard (44) using the technique of bone tissue culture demonstrated these effects quite clearly. Incubation of explanted mouse bone-rudiments with relatively high concentrations of parathyroid extract (Lilly) produced well-defined histological changes in bone. Microscopic examination of the bone shaft revealed a loss of osteoblasts, an increased number of osteoclasts, and active resorption. Raisz (91) confirmed these observations using bone rudiments pre-labeled with calcium-45. The calcium of the medium in which these rudiments were incubated with parathyroid hormone, was consistently higher indicating that the hormone was producing active resorption in tissue culture. Histologically, this resorption was associated with osteoclastic proliferation and decreased osteoblastic activity.

Talmage and Elliott (105) by applying the technique of

peritoneal lavage, demonstrated that parathyroidectomized rats could mobilize calcium from bone at the same rate as do normal rats. They concluded that the site of action of parathyroid hormone must be on the deeper trabeculae and not on the most recently deposited calcium. There is general acceptance that the regulation of blood calcium is physiologically a two-fold process: blood - bone ion transfer which is responsible for minute to minute homeostasis and a slower acting parathyroid hormone component which is responsible for hour to hour homeostasis. The blood - bone ion transfer affects only the labile calcium which occurs in newly forming and surface areas of bone while the parathyroid function affects the stable calcium located in the well-established mineralized areas of bone.

It has been shown that parathyroid extract and the metabolism of citric acid are intimately associated. Bone is the principal body resevoir of citric acid. Citrate analysis of alcohol extract of bone obtained from parathyroid extract-treated and normal animals, revealed a significant increase in citrate extractable from the bone of parathyroid extract-treated animals (36). Others (77) have noted an increase in radioactive citrate from pyruvate-2-^{14}C during the incubation of freshly isolated bone fragments obtained from parathyroid extract-treated animals. The data was interpreted to mean that parathyroid extract stimulates the formation of citrate from its precursors.

However, *in vitro* studies by Vaes and Nichols (100, 113) indicate that the metabolism of citrate in mouse metaphysis occurs at about ten times the rate of synthesis. Since bone cells have the capacity to metabolize the acid more rapidly than it can be formed, it would be more likely that the parathyroid extract effect in bone is on metabolism rather than synthesis. Bone cells can indeed synthesize citric acid, but elevated levels of citrate obtained upon parathyroid extract administration cannot be entirely attributed to increased synthesis; they are probably due to a blocking of citrate metabolism in some manner.

There have been several reports on the influence of parathyroid extract on bone metabolism using the techniques of both short term and long incubations. Cohn (27) and Cohn and Forscher (26) have studied the production of labeled carbon dioxide from Krebs cycle and glycolytic intermediates by rabbit femur slices prepared from parathyroid extract-treated and control animals. The production of labeled carbon dioxide from Krebs cycle intermediates such as citrate, succinate, and fumarate was reduced significantly by femurs of the treated animals. However, the release of labeled carbon dioxide from glucose was increased significantly by bone slices obtained from parathyroid extract-treated animals.

Mecca et al. (78) studied the same parameters as did Cohn but used a system of embryonic bone in culture for several days

with varying amounts of parathyroid extract. The metabolism of labeled citrate in parathyroid extract-treated cultures was depressed in comparison with control preparations. However, similar studies utilizing radioactive glucose and pyruvate as substrates showed no effect with respect to the release of labeled carbon dioxide. They also prepared homogenates from calvaria cultures and used them to study the release of labeled carbon dioxide from citrate. No differences were detected between control and parathyroid extract-treated calvaria homogenates until nicotinamide adenine dinucleotide phosphate (NADP) was added to the bone preparation. With this addition a decrease in the release of labeled carbon dioxide from parathyroid extract-treated calvaria was noted. The addition of NADP was probably required because the preparation of the homogenate no doubt disrupted the cellular organization, and not because of any altered parathyroid extract-induced effect produced in the homogenate.

There are then two distinct cellular processes in bone which have been observed experimentally to be influenced by parathyroid extract: bone resorption and bone metabolism.

Resorption is a complex process involving the dissolution of both mineral and organic matrix. There is no agreement as to which is initially removed. The removal of the organic matrix is in all probability an enzymic process, and there are two ways at least in which parathyroid hormone may control it: by

inducing the synthesis of new enzymes, or by activating latent ones already present in some inactive form. There is some evidence to support each viewpoint.

Evidence in favor of the first alternative are the observations that the administration of inhibitors of RNA or protein synthesis (actinomycin D or puromycin) inhibits the bone calcium mobilizing effects of parathyroid hormone *in vivo* (69, 96, 106) and *in vitro* (44, 91). *In vitro* studies by Raisz (92) indicate the parathyroid hormone-induced release of calcium is biphasic: an early release which is not inhibited by actinomycin D and a later progressive release which is actinomycin D sensitive. Rasmussen (96) substantiated this observation *in vivo* when he observed that parathyroid hormone administration to actinomycin D-treated animals caused an early rise in plasma calcium which was not sustained as in control animals. These data suggest that this hormone has an immediate effect upon bone resorption which does not involve a change in enzyme synthesis and a later effect which does. Raisz (92) also reported an apparent competition between actinomycin D and parathyroid hormone by demonstrating that increasing the concentration of parathyroid hormone overcomes the actinomycin D inhibition. He has suggested that the two agents compete for a binding site on DNA.

Vaes (114) has presented evidence which indicates that bone resorption may be brought about by the activation of latent enzymes. He has shown that bone cells contain membrane-bound

cytoplasmic organelles called lysosomes, which contain a number of acid hydrolases capable of participating in the hydrolysis of bone matrix. Activation of the lysosomal-bound enzymes would require only the rupture of the membrane. Vaes has demonstrated that at least some of these enzymes are released by bone in tissue culture under the influence of parathyroid hormone. His premise is that in a suitable acid environment resulting from the accumulation of organic acids, hydrolysis of the matrix can readily occur. He proposes that the action of parathyroid hormone is similar in this respect to that of vitamin A which has been shown to promote the release of lysosomal enzymes in cartilage (39).

To understand the mechanism of parathyroid hormone action it is necessary to determine the effects of the hormone at the cellular level, especially in light of evidence linking parathyroid hormone and RNA synthesis. This is extremely difficult to show in bone, mainly because of the low cell density and the marked heterogeneity of the cell population in this tissue. Consequently, studies of this type have been approached using homogeneous cell preparations (19, 111).

Borle and Neuman (19) have shown that HeLa cells grown in monolayer cultures resorb dead bone chips when incubated with parathyroid extract. Morphological changes in cell membrane, a decreased cellular calcium, and decreased adhesiveness of the cells to glass were also noted as a result of parathyroid

extract treatment. Similarly, Tenenhouse, Meier, and Rasmussen (111) have demonstrated that Ehrlich acites cells respond to parathyroid hormone. A two phase system was utilized in which cells were first incubated with the hormone, then the supernatant from the first phase was incubated with ^{45}Ca-labeled non-viable bone powder. The release of ^{45}Ca was used as a measure of parathyroid hormone activity. As little as 1 ug per ml of parathyroid hormone caused a significant increase in ^{45}Ca release. In this particular system, there is no contact between cells and bone powder. The stimulation of calcium release is therefore the result of the action of some factor released by the cells under the influence of parathyroid hormone. This effect is prevented by the addition of actinomycin D to the first phase. This observation suggests that RNA and protein synthesis are required for the production of the calcium-releasing factor.

It is possible that bone lysosome, protein synthesis, and metabolism are intimately connected in the parathyroid hormone function sequence. Parathyroid hormone causes the release of latent and/or synthesis of bone destroying enzymes. The accumulation of organic acids attributed to parathyroid hormone activity will maintain an acidic media at the resorptive site which in turn ensures the optimal activity of the bone destroying acid hydrolases. Besides ensuring optimal enzyme activity, the accumulation of acid products, particularly

citrate as suggested by Neuman and Neuman (82) would lead to the physical (non-enzymatic) dissolution of bone mineral. The citrate ion is a potent chelator of calcium ion as well as being capable of ionizing three moles of hydrogen per mole of citrate anion. Blood citrate concentrations are sensitive to the parathyroid status of the animal. L'Heureux and Roth (72) reported increases in both serum calcium and citrate after injection of parathyroid extract to normal rats. Parathyroid function dependent concentrations of bone lactate and one carbon acids also play a significant role in the resorptive acidification process.

THYROCALCITONIN

History and Origin

The events leading to the discovery of a hypocalcemic principle believed to be involved in blood calcium homeostasis were set in motion early in 1961. Copp (30) observed that injections of certain preparations of commercial parathyroid extract produced an initial hypocalcemic response prior to the expected hypercalcemia in the test animal. Perfusion of the parathyroid-thyroid apparatus in the dog with hypercalcemic blood resulted in an overall decrease in serum calcium (31). Copp concluded that the parathyroid gland was the source of a hypocalcemic principle which he named "calcitonin", since removal of the parathyroid gland before perfusion abolished the observed response. Kumar et al. (67) originally presented evidence which confirmed the parathyroid origin of the hypocalcemic agent, but later indicated that the thyroid was the primary source of calcitonin (68).

There is now a general consensus with the premise originally presented by Hirsch (55) that the thyroid gland is the principal source of a hypocalcemic hormone named thyrocalcitonin (TCT) as opposed to the postulated parathyroid hypocalcemic factor, calcitonin (13, 20, 45, 47, 112). Thyrocalcitonin has been isolated in quantities sufficient for assay from the thyroid glands of several species (47) including the rat, dog, goat, ox, monkey and human (1,3, 53, 56).

Hagis (48) by means of immunofluoresence technique, detected thyrocalcitonin in the cytoplasm of all epithelial cells of porcine thyroid glands. Similar techniques applied to porcine parathyroid glands did not detect the presence of thyrocalcitonin. Pearse et al. (87) have postulated that the thyroid C cells manufacture and store thyrocalcitonin in a form that is available for rapid release.

Recent developments indicate the possibility of the existence of two different hypocalcemic factors in the calcium homeostatic system calcitonin from the parathyroid gland (4, 21) and thyrocalcitonin from the thyroid gland.

Assay

There is no standard unit of thyrocalcitonin activity, but rather such units are arbitrarily defined in accordance with a dose-response relationship of the particular assay procedure under consideration (56, 68, 103). Most procedures measure the fall in serum calcium as the parameter for assay. The Medical Research Council of Britain in an effort to establish a uniform unit of activity has prepared for distribution vials containing a thyrocalcitonin preparation of a defined potency of one M.R.C. unit (56). The most salient characteristic observed in thyrocalcitonin assay is the rapid onset of a decrease in serum calcium of the test animals. In fusion of 50 mug/min into the tail vein of a 100 gram albino rat will produce a 1% decrease in serum calcium within 60 minutes (68).

Chemistry

Thyrocalcitonin is prepared from acid extracts of thyroid glands, usually porcine thyroid glands (47, 53, 85). The acid extract is subjected to solvent and salt fractionation and gel filtration to produce a highly purified product. Porcine thyrocalcitonin obtained by four different approaches using the above procedure (53) is a single chain polypeptide of a molecular weight range 5,200 ± 800. An appreciable amount of biological activity of porcine thyrocalcitonin is lost upon treatment of the hormone with hydrogen peroxide, and n - bromo-succinamide (108).

Biological Response and Mode of Action

Infusion of thyrocalcitonin in rats will produce hypophosphatemia concomitant with hypocalcemia as well as a passive hypocalciuria and a short term hyperphosphaturia (88, 98). Thyrocalcitonin markedly inhibits bone resorption both in vitro and in vivo (43, 45, 57, 60, 63, 75, 76). Histological examination of bone cultures incubated with parathyroid hormone and thyrocalcitonin, demonstrated that thyrocalcitonin can arrest parathyroid hormone induced bone resorption (43, 45). Injection of thyrocalcitonin into animals whose bones are pre-labeled with ^{45}Ca produces a decrease in the turnover rate of ^{45}Ca in both the labile and stable fractions of bone calcium (57, 60, 63). However, the inhibition is more pronounced in the

stable fraction of bone (57). Using the technique of lavage removal of calcium from bone, Klein et al. (62) observed that thyrocalcitonin produced a marked supression in the removal of calcium. These observations implicate bone and possibly the kidney as target organs of thyrocalcitonin. Another potential target organ of calcium homeostasis, the gastro-intestinal tract, has been shown to be unnecessary for thyrocalcitonin action (2). Studies on the influence of thyrocalcitonin on the lumen to plasma ion flux of isolated gut segments demonstrated a statistically insignificant decrease in flux (66). Recycling a fluid of known calcium concentration through the gut loops of anesthetized dogs injected with thyrocalcitonin produced a rapid fall in serum calcium but no change in gut calcium concentration (65). The question whether the kidney is a target organ at all remains controversial. Reports can be culled from the literature which cover an entire spectrum of results with respect to phosphate excretion and thyrocalcitonin. Some workers (79, 99, 100) reported experiments which detected a definite relationship between thyrocalcitonin and hyperphosphaturia. Others (98) have demonstrated a short term hyperphosphaturia, while Pechet et al. (88) report that thyrocalcitonin injection produces decreased excretion of both calcium and phosphate in rats.

Since parathyroid hormone and thyrocalcitonin have directly opposite responses with respect to bone resorption, one might

expect that thyrocalcitonin inhibits the secretion of or in some way inactivates parathyroid hormone. This does not seem to be the case since thyrocalcitonin is effective in parathyroidectomized animals (2, 55) and there is no evidence for the thyrocalcitonin inactivation of parathyroid hormone (2). The primary site of hypocalcemic action of the hormone appears to be at some stage in the resorptive process of bone (62). Thyrocalcitonin and parathyroid hormone probably do not compete at the same site in the resorptive process (55), but rather if one considers bone resorption a stepwise process, thyrocalcitonin exerts its effect at a step occurring earlier in the resorptive process to that site which is sensitive to the hypercalcemic hormone, parathyroid hormone.

BONE METABOLISM IN VITRO

Although bone contains several heterogeneous and quite distinct cell types it is believed that the metabolic pathways in these cells are basically the same. The overall pattern of glucose metabolism in freshly isolated bone differs very slightly from that pattern of glucose metabolism which would be found in any typical cell (26). There is a difference in the primary metabolic end product of bone cell metabolism and that of ordinary tissue cells. The fact that 50-60% of the metabolized glucose is converted into lactic acid is believed to be a reflection of the low oxygen tension in bone cells caused by the isolation and sluggish circulation in the Haversian Systems. Freshly isolated bone in a simple buffer exhibits an anaerobic pattern of glucose metabolism (17, 26) even in the presence of oxygen.

The anaerobic nature of bone metabolism perhaps lessens the importance of Krebs cycle activity, but it does not entirely obliterate its importance. The classic acid resorption theory of bone relies on the ability of citrate as a potent chelator of calcium ion. Citric acid was believed to play an important role in parathyroid hormone regulation of bone resorption (82). Most of the lactic acid formed in bone will eventually be metabolized via the Krebs-citric acid cycle.

The metabolism of bone is not exclusively limited to organic acids. Skeletal tissue contains considerable lipid of diverse

types (37, 71). It has been shown lipids can bind both calcium and phosphate (9, 40, 61). However, the exact role of lipid in bone is not known. A lipid material has been demonstrated at the sites of active calcification (58). This same unknown lipid material disappears from rachitic bone only to reappear when calcification is stimulated by vitamin D. The lipid material was not as demonstrable in older more calcified skeletal tissue.

Cruess and Clark (33) noted that the lipid composition of bone was altered by toxic doses of vitamin D or vitamin A. Administration of vitamin A decreased the total fatty acid content of cortical bone while vitamin D was observed to increase triglycerides, esterified cholesterol and phospholipids in bone.

Protein and amino acid metabolism in bone are important in the formation of the organic collagen matrix. Collagen synthesis in isolated bone segments will continue for at least eight hours in vitro (34, 41). Collagen is formed in cells and is released as a relatively stable component of the organic matrix. Bone matrix synthesis is oxygen dependent, since collagen synthesis is stimulated by glucose and inhibited by anaerobiosis.

There have been two general approaches in the study of bone metabolism in vitro; namely, the use of freshly isolated bone segments and the use of bone tissue cultures. Each of these techniques have inherent advantages and disavantages.

Freshly isolated bone fragments are usually employed in short term experiments. The short term aspect allows a precise control of incubation medium and allows one to study tissue performing a wide variety of functions; glycolysis, amino acid transport, fat synthesis and the formation of collagen. The less durable cells of the isolated fragments die soon after isolation. The overall viable state appears to be 6 - 12 hours in which the initial metabolism of surviving bone approximates the true in vivo state of metabolism.

The prolonged term incubation of bone cell cultures involves complex factors. The consistency of the media necessary to support such an incubation is quite difficult to control owing to the presence of inumerable constituents. Most synthetic tissue culture media require the addition of serum. This necessitates obtaining a uniform supply of serum from a single source in order to prepare consistent incubation media over a period of time.

Statement of the Problem

There is considerable evidence which indicates that parathyroid hormone (PTH) and thyrocalcitonin (TCT) have a direct action on bone, the principal target organ of these two calcium regulating hormones. Numerous reports have appeared concerning the relationship between parathyroid extract (PTE) and the metabolism of glucose and organic acids in bone. However, purified parathyroid hormone does not influence metabolism *in vitro* (52) as indicated in the Ehrlich ascites carcinoma cell assay. This assay measures the flux in production of $^{14}CO_2$ from glucose-6-^{14}C or pyruvate-3-^{14}C in tumor cells incubated with peptides of variable metabolic activity. PTE and non-calcium mobilizing fractions obtained during the purification of PTE will stimulate the production of $^{14}CO_2$ from the aforementioned substrates but purified PTH has no effect. There have been no reports to date concerning the relationship between the thyrocalcitonin and the oxidation of substrates.

It is currently believed that the metabolism of organic acids in bone may not constitute a direct role in the mechanism of action of calcium homeostasis as previously proposed (82), but rather the release and synthesis of bone destroying enzymes (114) may be the primary initiators of the hypercalcemic response.

The postulate of Neuman and Neuman (82) indicated that PTH produced its hypercalcemic action by increasing localized

acidity in bone via PTH-induced alteration in the rate of accumulation of acid products of bone metabolism which would dissolve bone mineral. PTH could increase acidity at the resorptive site by either increasing the metabolism of glucose to organic acids and CO_2 or by blocking the oxidation of organic acids so as to cause their accumulation. However, recent evidence indicates that bone resorption is not quite as simple as the process outlined by Neuman and Neuman. Many enzymes have been shown to be involved in resorption (114). PTH action has been shown to be dependent on m-RNA synthesis (91) indicating that the synthesis of protein, probably enzymes, is involved in the mechanism of action of PTH.

Since the hypercalcemic principle, parathyroid hormone, does not affect oxidation of substrate <u>in vitro</u>, it would be of interest to discover the agent which does produce the fluctuations in metabolism of glucose and organic acids commonly associated with the injection of PTE and PTH, and the relative importance of these functions with reference to the mechanism of action of the hypercalcemic response. Also, thyrocalcitonin has a direct effect on bone (2) and is a PTH antagonist (63) on many levels of PTH action. Information concerning the unexplored relationship between TCT and bone metabolism might be helpful in establishing the relative importance of alterations in the oxidation of glucose and organic acids in the overall mechanism of calcium homeostasis.

The approach to the problem will be a modification of the one employed by DeLong and L'Heureux (35). The bone preparations will be used to relate the hormonally-induced metabolic phenomena (thyrocalcitonin and parathyroid extract induced alterations in the evolution of CO_2 from substrates) with the hormonally-induced physiological and histological phenomena (parathyroid extract and thyrocalcitonin altered levels of serum calcium).

Two of the anatomically distinct regions of femur will serve as tissue specimens; the diaphysis and epiphysis. These two regions were chosen because of marked differences in physical structure and cell distribution. It is possible that these differences may be reflected in the action of PTE and TCT on these tissues.

Also the influence of calcium ion on the oxidation of substrate will be studied in an effort to establish a direct relation between substrate oxidation rate and calcium ion concentration *in vitro* and *in vivo*.

CHAPTER II

MATERIALS AND METHODS

General Experimental Procedure

Male Holtzman albino rats weighing approximately 140 - 170 grams were thyroparathyroidectomized in order to remove the influence of endogenous parathyroid hormone and thyrocalcitonin activity. The animals were prepared in the following manners in order to study the desired experimental parameter.

1. Parathyroid extract treatment and bone metabolism. The animals received two injections; experimental animals received 75 U.S.P. units of Injection Parathyroid (Lilly) and control animals received a comparable volume of vehicle at 24 and 48 hours after surgery. The vehicle injected was that used in the commercial preparation of the extract and was composed of 1.6% glycerol, 0.2% phenol and 0.9% sodium chloride.

2. Calcium ion concentration and bone metabolism. In order to study *in vivo* calcium ion concentration and bone metabolism the animals were maintained on calcium deficient diet and normal diet for a period of one week. The *in vitro* effect of calcium ion on bone metabolism was studied by incubating bone tissue in media which contained various concentrations of calcium.

3. PTE, PTE-TCT treatment and bone metabolism. The animals were prepared as in procedure 1. However, twelve hours after the last injection of PTE a group of PTE treated

animals received subsequent injections of TCT over a two hour period.

In all the experiments, the bone tissue was prepared much in the same manner. A weighed portion of bone fragments or an aliquot of bone homogenate was placed into a 25 ml incubation flask which contained a suitable incubation medium. Fragments and homogenates were prepared from both the diaphysis and epiphysis of femurs obtained from all test animals. Radioactively-labeled substrate was added to each flask and the flask was sealed with a rubber septum in which is inserted a plastic centerwell containing a 10% solution of KOH. The incubation was carried out in a shaker water bath at a temperature of 37° C for a period of three hours.

After three hours the viable tissue was killed by the addition of 2 ml of 10% trichloroacetic acid to each flask. The 10% solution of KOH which was contained in the centerwell was transferred to a culture tube. The labeled carbon dioxide absorbed in the KOH solution was precipitated as barium carbonate and the radioactivity was determined.

SERUM CALCIUM DETERMINATION

A method of determining ionizable calcium in serum, which is essentially unaffected by organic materials present, has been reported by Ashby and Roberts (5). This method is based upon the fact that at pH's above 12, calcein (an iminoacetate

derivative of fluorescein) fluoresces under long-wave ultraviolet light only in the presence of free calcium. In an analysis, a given amount of ethylenediaminetetraacetic acid (EDTA) which is in excess of that required to complex all of the calcium present is added to the sample. A small amount of cyanide is added to complex any copper or iron which is present. The solution is back-titrated with standard calcium solution with the calcium replacing the magnesium in any magnesium-EDTA complex which might have formed. When all of the EDTA has become complexed with calcium, additional calcium will combine with calcein causing fluorescence, and the end point will have been reached. Titrations must also be performed on calcium-free samples in order to determine the total calcium binding capacity of the EDTA aliquots. The serum calcium level is determined from the difference between the total calcium binding capacity of the EDTA and the amount of calcium required to titrate the serum sample.

Four hundred microliters of blood sample were obtained from each rat by tail bleeding. The samples were placed in the refrigerator for a short time before the clot was separated by a five minute centrifugation in a Beckman/Spinco microfuge. Thirty microliter aliquots of serum were added to microtitrator cups. To each cup was added 0.150 ml of 0.001 M EDTA, one drop (about 60 ul) of dilute calcein indicator, 1 drop (about 30 ul) of 1% sodium cyanide solution, and 1 drop (about 60 ul) of 1.0

N sodium hydroxide. Titration was performed with a micro-titrator containing a 20.0 mg% standard calcium solution. A long-wave ultraviolet lamp (Mineralight, Model SL 3660) was placed about two inches above the titration cup. The standard calcium solution was added with continuous stirring until the green fluorescence which was observed no longer increased in intensity. The calcium concentration of the sample is directly proportional to the difference in microliters of standard calcium required to titrate the sample and a water blank. A 10.0 mg% standard calcium solution was run with each group of samples and the calcium concentrations were calculated as follows:

$$\text{mg\% calcium} = 10 \text{ mg\%} \times \frac{\text{microliters for unknown}}{\text{microliters for 10 mg\% standard}}$$

Where:

microliters unknown = microliters of titrant required to titrate blank minus microliters of titrant required to titrate unknown sample

microliters 10 mg% standard = microliters of titrant required to titrante blank minus microliters of titrant required to titrate 10 mg% standard

Figure I indicates the linear relationship between the microliters of titrant used and the concentration of calcium in solution. This is the standard calcium titration curve used to

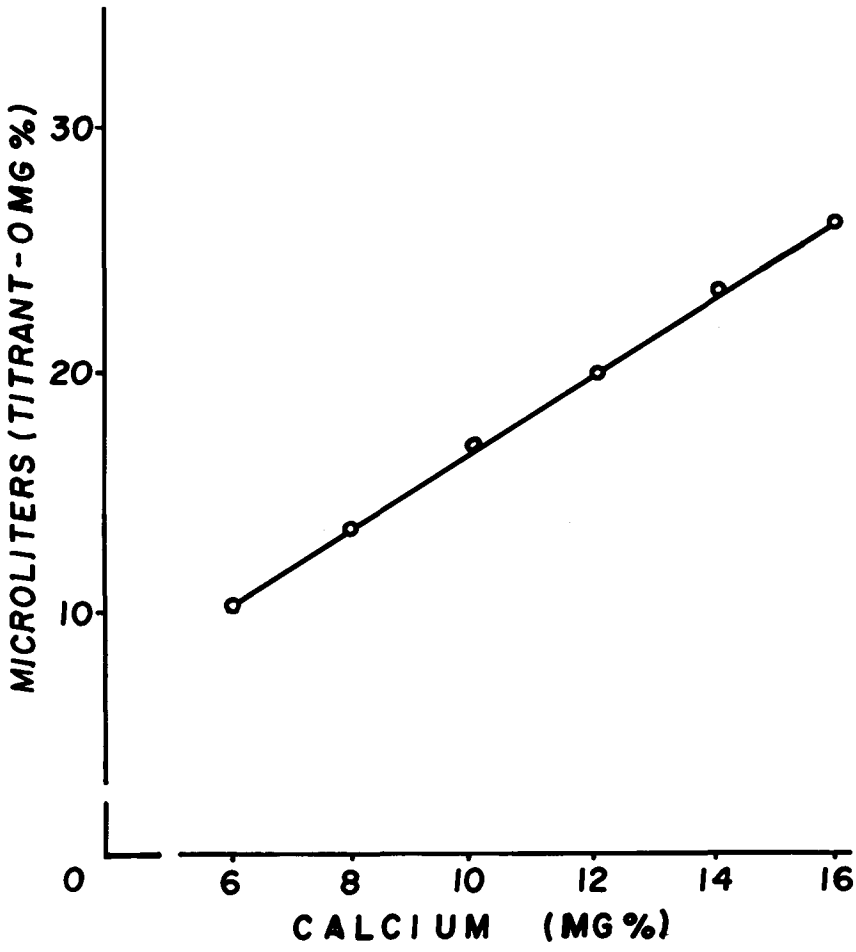

FIGURE I

STANDARD CURVE FOR CALCIUM DETERMINATION

TABLE I

STANDARD TITRATION CURVE DATA FOR CALCIUM DETERMINATION

Actual Calcium	Number of Samples	Microliters of Titrant	Microliters (Titrant - 0 mg%)
0 mg%	6	30.7 ± 0.2	
6	6	20.6 ± 0.3	10.2
8	6	17.3 ± 0.4	13.4
10	6	13.9 ± 0.3	16.8
12	6	10.8 ± 0.5	19.9
14	6	7.5 ± 0.2	23.3
16	6	4.9 ± 0.3	25.9

calculate serum calcium concentration for the animals used in the experiments. The data for this standard curve appears in Table I.

Solutions:

Calcein solution-concentrated. 0.25 g of powdered indictor was dissolved in 4.0 ml of 1.0 N NaOH. When solution was complete this was diluted to 100 ml with doubly distilled water.

Calcein indicator solution. 0.5 ml of the concentrated calcein solution was diluted to 25 ml with doubly distilled water.

Calcium standard solution. 0.2497 g of oven-dried calcium carbonate was dissolved in 30 ml of 2 N HCl and diluted to 500 ml with doubly distilled water. Working solutions were prepared from this stock solution.

Ethylenediaminetetraacetic acid standard. 0.375 g of EDTA dissolved in CO_2-free water to a total volume of 1000 ml.

BLOOD SAMPLING:

Blood samples were obtained from the tail of the rat. The rat was placed in a restraining cage and the tail severed approximately three quarters of an inch from the tip. Bleeding was aided by "milking" the tail in a proximal to distal direction. Blood was collected in plastic microtubes, and these were allowed to stand at room temperature for thirty minutes to one hour to allow for clot formation and retraction.

Centrifugation was performed in a Beckman Spinco/Microfuge for two minutes. Supernatant serum was separated from centrifugate and stored under refrigeration in a microtube until analyzed.

THYROPARATHYROIDECTOMY:

Thyroparathyroidectomy at birth results in a severe retardation of growth and skeletal development. The changes are less severe if the operations are performed at later ages and usually require a period of weeks to become manifest.

The surgical technique employed in the thyroparathyroidectomy of male albino rats is one commonly employed in numerous laboratories. Animals 120 - 140 grams in weight are anesthetized with a dose of aqueous nembutal solution in the proportion of 33 mg nembutal/Kg of body weight. The rat is secured to an operating board and the head is kept extended by means of a rubber band which is looped over the upper incisors. A ventral midline skin incision is made extending slightly beyond the upper and lower boarders of the submaxillary gland. The salivary glands are gently teased aside as any unnecessary trauma will cause severe bleeding and the underlying musculature opened at the midline by blunt dissection. The musculature immediately surrounding the treachea is teased apart at the midline and retracted, thus exposing the two lobes and the isthmus of the thyroparathyroid apparatus. The glands are then carefully teased from the treachea, preferably intact (two lobes connected by the isthmus) to ensure completeness of removal.

After the removal of the glands, a wedge of sterile cotton is placed over the wound until the bleeding is assuaged. The cotton is removed and the wound is closed by the use of Michel wound clips.

The animals require from three to six hours to recover from the anesthesia. Those animals which developed difficulties in breathing or severe loss in weight within two days were not used in the experiments. During the operation, clean but not necessarily aseptic technique was employed. The rat does not readily become infected in the region of the neck. Overall, male albino rats within a weight range of 120 - 140 grams seem to respond better to surgery than heavier animals. In animals weighing less than 100 grams, the thyroid-parathyroid apparatus is often difficult to find.

THE PREPARATION OF FRAGMENTS AND HOMOGENATES FROM RAT FEMUR

Male Holtzman rats were stunned by a sharp blow to the head and decapitated. A semicircular incision was made around the leg just below the pelvis. The skin was pulled toward the foot exposing the musculature of the thigh area. The tendon connecting the epiphysis and a portion of the musculature was severed. The severed tendon was grasped with forceps and pulled toward the pelvis. This procedure tears away most of the ventral musculature of the thigh and exposes the femur. The remaining musculature was scraped away with a scalpel. The

femur was severed from the tibia and worked free from the socket in the pelvis.

The extirpated femur was placed in ice-cold incubation media. The bone was cleaned of all adherent muscle, periosteum, and cartilage. The distal epiphysis is separated from the diaphysis. The proximal epiphysis is also separated from the diaphysis, but it is discarded. The distal epiphysis and diaphysis were split longitudinally and the marrow removed by scraping with the point of a scalpel and washing with isotonic saline.

For the preparation of bone fragments, the sections of epiphysis and diaphysis were blotted dry and weighed on a Roller-Smith balance. Approximately 150 - 200 mg of the split bone sections were fragmentized on glass or lucite plate using a stainless steel scalpel and placed in a twenty-five ml incubation flask containing 4.0 ml of incubation media.

For the preparation of homogenates, sections of epiphysis and diaphysis were placed in a bone disintegrinder (Kontes) with 10.0 - 30.0 ml of incubation media depending on the amount of bone to be homogenized. Homogenization was performed for five minutes or until no further contact between the disintegrinder and bone could be felt.

Homogenates have a milky appearance and settle out rapidly. While pipetting, the homogenate is kept agitated with a magnetic stirrer. Whenever possible tissue was maintained at

a temperature of 0° C during preparation.

An estimation of the amount of material in any particular volume of homogenate was obtained by pipetting a 1.0 ml aliquot of homogenate on a preweighed planchet. The aliquot was dried on the planchet using a heat lamp. The planchet was weighed and the dry weight of the material in the aliquot determined by the weight difference of the planchet. This was necessary so that the data could be expressed according to $^{14}CO_2$ evolved from substrate metabolized by an estimated amount (dry weight) of bone present in the homogenate.

PREPARATION OF INCUBATION MEDIUM

The medium used in these experiments was prepared by mixing 232 ml. 0.154 M NaCl, 8 ml. 0.154 M KCl, 6 ml. 0.110 M $CaCl_2$, and 2 ml 0.154 M $MgSO_4$, and adding 25 ml of phosphate buffer prepared by bringing 75 ml. of 0.2 N NaOH to a pH of 7.4 with 2 M H_3PO_4 and diluting to 100 ml. with doubly distilled water. All solutions were prepared with doubly distilled water.

LABELED CARBON DIOXIDE COLLECTION AND MEASUREMENT

Radioactive carbon dioxide produced by the oxidation of the radioactively labeled substrate in the bone tissue preparation was absorbed in 0.2 ml of a 10% solution of potassium hydroxide contained in the centerwell of the incubation flask. The centerwell of the flask also contained a small fold of

Whatman #1 filter paper. The purpose of the paper was to increase the surface area of contact of the potassium hydroxide solution and to help control movement of the liquid during the incubation in the shaker bath.

After the incubation period, the filter paper in the centerwell was transferred to a test tube. Five ml of CO_2 free distilled water was added to each tube, and the contents of the tubes were transferred quantitatively to another set of culture tubes which each contained 5 ml of 10% barium chloride solution. The tubes were allowed to stand at room temperature until the white, flocky, precipitate settled.

The tube contents were transferred into a Tracerlab precipitation Apparatus, Model E-8B which was fitted with a disk of Whatman #50 filter paper. Suction was applied and the radioactive barium carbonate was plated on the disk of filter paper. The plated precipitate was washed successively with water and dried with acetone. The plated samples were allowed to dry for two hours before weighing and counting.

The counting was accomplished with a thin end-window type assembly consisting of a Tracerlab Tracermatic SC-83 Scaler, Tracerlab SC-100 Multi-Matic Sample Changer, and a Tracerlab SC-66 Printing Timer. Corrections for self-absorption (25) were determined by establishing a $Ba^{14}CO_3$ self-absorption curve (Figure 2). The tabulated data for the determination curve appears in Table II.

TABLE II

$Ba^{14}CO_3$ SELF-ABSORPTION CORRECTION VALUES

$Ba^{14}CO_3$ mg	Correction	$Ba^{14}CO_3$ mg	Correction
0.0-0.5	1.00	14.0-14.5	0.52
0.5-1.0	0.95	14.5-15.0	0.51
1.0-1.5	0.93	15.0-15.5	0.50
1.5-2.0	0.91	15.5-16.0	0.49
2.0-2.5	0.89	16.0-16.5	0.48
2.5-3.0	0.88	16.5-17.0	0.47
3.0-3.5	0.86	17.0-17.5	0.46
3.5-4.0	0.82	17.5-18.0	0.45
4.0-4.5	0.80	18.0-18.5	0.44
4.5-5.0	0.78	18.5-19.0	0.43
5.0-5.5	0.76	19.0-19.5	0.43
5.5-6.0	0.74	19.5-20.0	0.42
6.0-6.5	0.73	20.0-20.5	0.42
6.5-7.0	0.71	20.5-21.0	0.41
7.0-7.5	0.70	21.0-21.5	0.41
7.5-8.0	0.68	21.5-22.0	0.40
8.0-8.5	0.67	22.0-22.5	0.39
8.5-9.0	0.66	22.5-23.0	0.39
9.0-9.5	0.65	23.0-23.5	0.38
9.5-10.0	0.64	23.5-24.0	0.38
10.0-10.5	0.62	24.0-24.5	0.37
10.5-11.0	0.61	24.5-25.0	0.37
11.0-11.5	0.60	30.0	0.32
11.5-12.0	0.58	35.0	0.29
12.0-12.5	0.57	40.0	0.26
12.5-13.0	0.56	45.0	0.23
13.0-13.5	0.55	50.0	0.21
13.5-14.0	0.53		

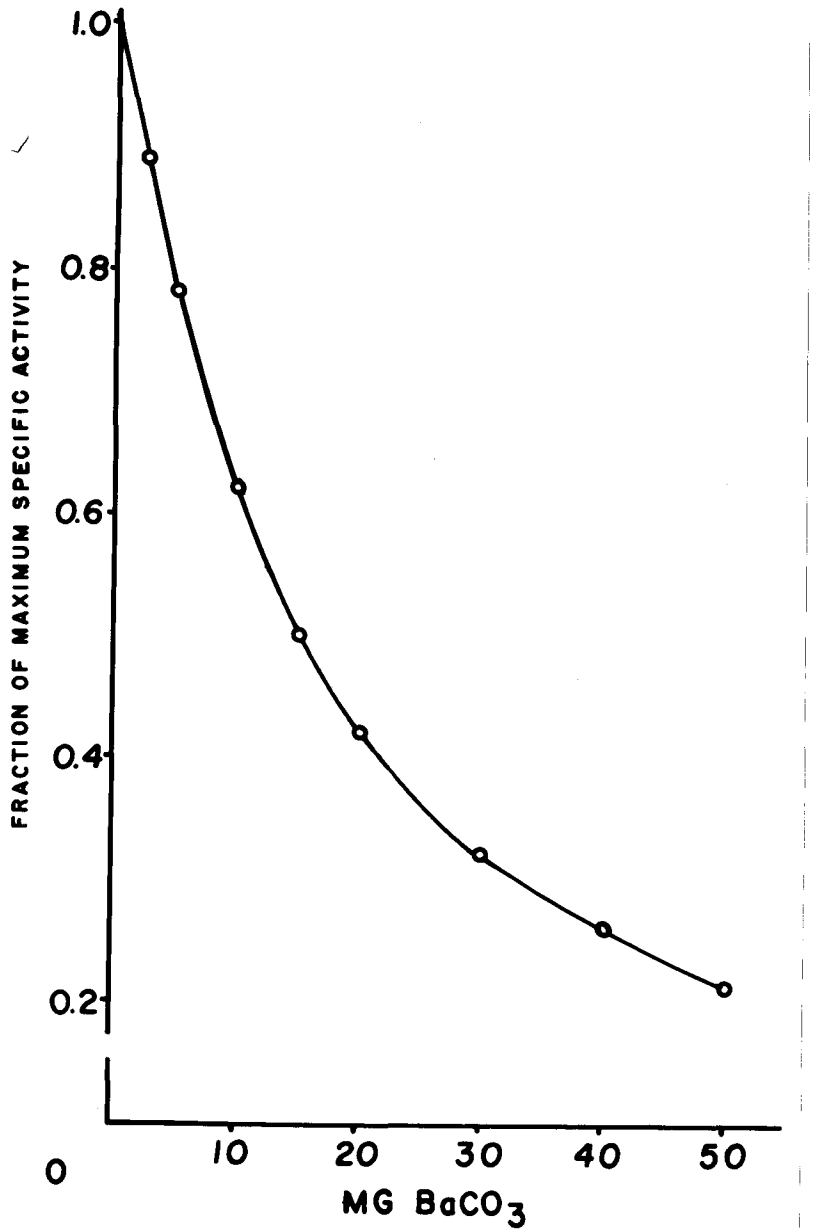

FIGURE 2

SELF-ABSORPTION CORRECTION CURVE FOR $Ba^{14}CO_3$

CHAPTER III

EXPERIMENTAL RESULTS

The experiments utilized the detection of $^{14}CO_2$ evolution from the radioactively labeled substrate as a measure of the *in vitro* oxidation of substrate by bone tissue preparations. The substrates used in the experiments included citrate-1,5-^{14}C, succinate-2,3-^{14}C, 2-oxoglutarate-5-^{14}C, and pyruvate-2-^{14}C. The oxidation of these substrates was studied using fragments and NADP fortified homogenates prepared from the diaphysis and epiphysis of femurs excised from the various test animals.

The data are presented for each flask used in the incubation series. The parathyroid status of the experimental and control animals was assessed on the basis of their respective serum calcium levels. The blood samples were obtained immediately prior to sacrifice and analyzed later for calcium concentration. The elapsed time between sacrifice of the animals and the beginning of the experiment ranged from 2 - 3 hours, during which time the tissue was prepared for incubation.

Experiment 1: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Citrate-1,-5^{14}C.

Six thyroparathyroidectomized rats were used in the experiment. Three of the six were injected subcutaneously with parathyroid extract and three with vehicle. The rats which

received 150 U.S.P. over a period of two days showed an average increase in serum calcium of 2.9 mg%.

In the experiment, 1.0 microcurie of radioactive substrate, 150 - 200 mg of bone fragments and 4.0 ml of incubation media were present in each flask. The incubation was carried out for two hours at 37° C.

The results of the experiment appear in Table III. The diaphysis of the femur showed a depression in the oxidation of substrate by the bone preparation obtained from the PTE-treated animals. The epiphysis of the femur exhibited an increase in the oxidation of the radioactive substrate by the bone preparations obtained from the PTE-treated animals.

Experiment 2: The Incubation of Fragments of Femur Diaphysis and Epiphysis from PTE-Treated and Control Animals with Succinate-2,3-^{14}C.

Eight thyroparathyroidectomized animals were used in Experiment 2. Four of the rats received subcutaneous injections of 150 U.S.P. units of parathyroid extract over a period of two days. The control animals received a comparable injection of vehicle. The PTE-treated animals had an average increase in serum calcium of 2.7 mg%.

Two microcuries of succinate-2,3-^{14}C, 200 mg of bone fragments and 4.0 ml of incubation media were present in each of the incubation flasks. The incubation was run for three and one-half hours at 37° C in a constant temperature shaker water

TABLE III

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY FRAGMENTS OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	22
Citrate-1,5-^{14}C	PTE-Treatment	31
Citrate-1,5-^{14}C	Control	52
Citrate-1,5-^{14}C	Control	49

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	19
Citrate-1,5-^{14}C	PTE-Treatment	23
Citrate-1,5-^{14}C	Control	13
Citrate-1,5-^{14}C	Control	10

bath.

The diaphysis of the femur showed a depression in the oxidation of substrate by the bone preparation obtained from the PTE-treated animals when compared to that of control animals. The epiphysis of the femur produced an increase in the oxidation of the radioactive substrate by the bone preparation obtained from the PTE-treated animals (Table IV).

Experiment 3: The Incubation of Fragments of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Pyruvate-2-^{14}C.

Six thyroparathyroidectomized rats were used in the experiment. Three of the animals were injected subcutaneously with 150 U.S.P. units of parathyroid extract and three were injected subcutaneously with vehicle over a period of two days. The rats which were injected with parathyroid extract showed an average increase in serum calcium of 2.8 mg%.

Two microcuries of radioactive substrate, 100 mg of bone fragments, and 4.0 ml of media were present in each incubation flask. The incubation was performed for three hours at 37° C.

It can be seen from the data presented in Table V that the diaphysis of the femur showed an increase in the evolution of $^{14}CO_2$ by the bone preparation obtained from the PTE-treated animals. The epiphysis also showed an increase in the evolution of $^{14}CO_2$ from the radioactive substrate by the bone preparation obtained from the PTE-treated animals.

TABLE IV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY FRAGMENTS OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE-Treatment	29
Succinate-2,3-^{14}C	PTE-Treatment	30
Succinate-2,3-^{14}C	PTE-Treatment	31
Succinate-2,3-^{14}C	Control	48
Succinate-2,3-^{14}C	Control	44
Succinate-2,3-^{14}C	Control	38

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE-Treatment	88
Succinate-2,3-^{14}C	PTE-Treatment	71
Succinate-2,3-^{14}C	PTE-Treatment	72
Succinate-2,3-^{14}C	Control	53
Succinate-2,3-^{14}C	Control	55
Succinate-2,3-^{14}C	Control	55

TABLE V

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY FRAGMENTS OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	382
Pyruvate-2-^{14}C	PTE-Treatment	370
Pyruvate-2-^{14}C	PTE-Treatment	367
Pyruvate-2-^{14}C	Control	264
Pyruvate-2-^{14}C	Control	222
Pyruvate-2-^{14}C	Control	203

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	780
Pyruvate-2-^{14}C	PTE-Treatment	800
Pyruvate-2-^{14}C	PTE-Treatment	920
Pyruvate-2-^{14}C	Control	564
Pyruvate-2-^{14}C	Control	574
Pyruvate-2-^{14}C	Control	610

Bone cells are distributed unevenly throughout the bone tissue. Ossification centers and growing areas of bone will have a denser cell population than older, completely mineralized areas of bone. In the preparation of bone fragments the possibility exists of inadvertently "loading" an incubation flask with tissue containing a preponderance of living cells and another with tissue containing mainly non-viable material. Also, the radioisotope must penetrate the intricate canals in the fragmentized Haversian system of the femur in order to come into contact with the viable cell. This means that production of $^{14}CO_2$ from labeled substrate will at least partially reflect the ability of the labeled substrate to penetrate into the cell.

Thus a method was devised to obtain a more physically uniform bone preparation with the use of a bone disintegrinder (Kontes). The homogenate prepared through the use of the disintegrinder was rather coarse and had to be kept agitated or the milky suspension would rapidly settle out. To measure the homogeneity in the various homogenates prepared, one ml aliquots were pipetted into preweighed planchets and dried. The dried aliquots had a weight variation of $\pm$ 0.20 mg in 15 mg, the weight of a 1 ml aliquot.

Experiments 4 and 5: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Citrate-1,5-^{14}C.

Four thyroparathyroidectomized rats were used in each experiment. Two rats were injected subcutaneously with parathyroid extract and the control animals were injected with vehicle. The animals which received 162 U.S.P. units of parathyroid extract over a period of two days showed an average increase in serum calcium of 2.1 mg% in Experiment 4 and 2.4 mg% in Experiment 5.

In Experiment 4, 0.5 microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask. The incubation was run for three hours at 37° C.

Femur diaphysis and epiphysis showed no differences in the oxidation of substrate by bone preparations obtained from the PTE-treated rats when compared to that of control preparations. (Table VI).

In Experiment 5, one microcurie of citrate-1,5-^{14}C and 4.0 ml of bone homogenate were present in each incubation flask. The incubation was performed for three hours at 37° C in a constant temperature shaker water bath.

Femur diaphysis and epiphysis showed no differences in the oxidation of substrate by bone preparation obtained from the PTE-treated animals when compared to that of control preparations (Table VII).

TABLE VI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	45
Citrate-1,5-^{14}C	PTE-Treatment	43
Citrate-1,5-^{14}C	Control	41
Citrate-1,5-^{14}C	Control	39

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	64
Citrate-1,5-^{14}C	PTE-Treatment	67
Citrate-1,5-^{14}C	Control	65
Citrate-1,5-^{14}C	Control	63

TABLE VII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	26
Citrate-1,5-^{14}C	PTE-Treatment	22
Citrate-1,5-^{14}C	Control	22
Citrate-1,5-^{14}C	Control	24

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	36
Citrate-1,5-^{14}C	PTE-Treatment	34
Citrate-1,5-^{14}C	Control	34
Citrate-1,5-^{14}C	Control	33

Experiment 6: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Succinate-2,3-^{14}C.

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with parathyroid extract and two were injected with vehicle over a period of two days. The animals which each received 162 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.9 mg%.

One half microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask. The incubation was run for three hours at 37° C.

No differences in the evolution of $^{14}CO_2$ were noted in either the diaphysis or the epiphysis prepared either from PTE-treated and control animals (Table VIII).

Experiment 7 and 8: The Incubation of Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE and Control Animals with Pyruvate-2-^{14}C.

Four thyroparathyroidectomized rats were used in each experiment. Two of the animals were injected subcutaneously with parathyroid extract and two were injected with vehicle over a period of two days. The rats which received 162 U.S.P. units of parathyroid extract showed an average increase in serum calcium of 1.8 mg% in Experiment 7 and 2.5 mg% in Experiment 8.

TABLE VIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE-Treatment	19
Succinate-2,3-^{14}C	PTE-Treatment	18
Succinate-2,3-^{14}C	PTE-Treatment	18
Succinate-2,3-^{14}C	PTE-Treatment	17
Succinate-2,3-^{14}C	Control	19
Succinate-2,3-^{14}C	Control	15
Succinate-2,3-^{14}C	Control	17
Succinate-2,3-^{14}C	Control	19

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE-Treatment	55
Succinate-2,3-^{14}C	PTE-Treatment	60
Succinate-2,3-^{14}C	PTE-Treatment	57
Succinate-2,3-^{14}C	PTE-Treatment	55
Succinate-2,3-^{14}C	Control	55
Succinate-2,3-^{14}C	Control	53
Succinate-2,3-^{14}C	Control	56
Succinate-2,3-^{14}C	Control	57

One-half microcurie of radioactive substrate and 4.0 ml of homogenate were present in each flask in Experiment 7. One microcurie and the same amount of homogenate were present in each flask in Experiment 8. The incubations were carried out at 37° C for three hours.

The diaphysis of the femur showed an increase in the oxidation of pyruvate-2-^{14}C by bone homogenate prepared from the PTE-treated animals. The epiphysis of the femur also showed an increase in the oxidation of substrate by bone prepared from the PTE-treated preparations. This pattern was observed in both Experiments 7 and 8 (Table IX and X).

It is apparent from the results (Experiments 4 - 6) that the homogenization procedure has a profound effect on the ability of the bone tissue preparation to detect hormonal treatment on a metabolic level, as indicated in the absence of alterations in the oxidation of organic acid. The preparations were viable in that $^{14}CO_2$ was produced from citrate-1,5-^{14}C and succinate-2,3-^{14}C, but no differences could be detected between the PTE-treated and control preparations of diaphysis and epiphysis. This finding is similar to that reported by Mecca et al. (78) in which case homogenized calvaria were viable but showed no differences in the oxidation of organic acids when incubated with PTE as the tissue did when intact calvaria were incubated with PTE.

However, comparable homogenate preparations incubated with

TABLE IX

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	780
Pyruvate-2-^{14}C	PTE-Treatment	810
Pyruvate-2-^{14}C	PTE-Treatment	960
Pyruvate-2-^{14}C	PTE-Treatment	840
Pyruvate-2-^{14}C	Control	570
Pyruvate-2-^{14}C	Control	580
Pyruvate-2-^{14}C	Control	580
Pyruvate-2-^{14}C	Control	550

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	1,380
Pyruvate-2-^{14}C	PTE-Treatment	1,400
Pyruvate-2-^{14}C	PTE-Treatment	1,590
Pyruvate-2-^{14}C	PTE-Treatment	1,390
Pyruvate-2-^{14}C	Control	1,320
Pyruvate-2-^{14}C	Control	1,200
Pyruvate-2-^{14}C	Control	1,200
Pyruvate-2-^{14}C	Control	1,300

TABLE X

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF BONE DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	1,220
Pyruvate-2-^{14}C	PTE-Treatment	1,100
Pyruvate-2-^{14}C	PTE-Treatment	1,160
Pyruvate-2-^{14}C	Control	840
Pyruvate-2-^{14}C	Control	700
Pyruvate-2-^{14}C	Control	740

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	3,250
Pyruvate-2-^{14}C	PTE-Treatment	2,800
Pyruvate-2-^{14}C	PTE-Treatment	2,900
Pyruvate-2-^{14}C	Control	2,200
Pyruvate-2-^{14}C	Control	2,300
Pyruvate-2-^{14}C	Control	2,300

pyruvate-2-^{14}C were sensitive to preparation differences in hormonal treatment (PTE vs. controls, Experiments 7 and 8). Mecca et al. found that the addition of the cofactor NADP to calvaria homogenates would increase viability of the homogenate and restore the sensitivity to PTE-treatment, in the oxidation of citrate-1,5-^{14}C. No other labeled substrates except citrate-1,5-^{14}C were used in this procedure.

In the next series of experiments, homogenates were prepared in the usual manner, except that a measured amount of NADP was added to each homogenate. This procedure was decided upon to determine if the addition of this cofactor to femur homogenate would restore viability and/or sensitivity of the bone preparation to PTE-treatment.

Experiment 9: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Citrate-1,5-^{14}C.

Four thyroparathyroidectomized rats were used in the experiment. Two of the animals were injected subcutaneously with parathyroid extract and two were injected with vehicle. The rats which received 100 U.S.P. units of injection parathyroid demonstrated an average increase in serum calcium of 3.0 mg% over the control animals.

One-half microcurie of radioactive substrate, 4.0 ml of homogenate and 16.0 micromoles of NADP were present in each flask. The incubation was carried out for three hours at 37° C.

The diaphysis of the femur demonstrated an increase in the oxidation of radioactive substrate by bone homogenate obtained from PTE-treated animals. The epiphysis of the femur also showed an increase in the oxidation of substrate by bone homogenate obtained from the PTE-treated animals (Table XI).

Experiment 10: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Succinate-2,3-^{14}C.

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats were injected subcutaneously with 100 U.S.P. units of parathyroid extract; two received a comparable volume of vehicle. The animals which received the parathyroid injection showed an average increase in serum calcium of 2.6 mg%.

One-half microcurie of radioactive substrate, 4.0 ml of homogenate, and 16.0 micromoles of NADP were present in each flask. The incubation was carried out for three hours at 37° C.

The diaphysis of the femur showed an increase in the oxidation of the substrate by the bone preparation obtained from the PTE-treated animals. The epiphysis of the femur demonstrated an increase in the oxidation of the substrate by bone preparations obtained from PTE-treated animals (Table XII).

TABLE XI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	126
Citrate-1,5-^{14}C	PTE-Treatment	123
Citrate-1,5-^{14}C	Control	53
Citrate-1,5-^{14}C	Control	59

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE-Treatment	385
Citrate-1,5-^{14}C	PTE-Treatment	365
Citrate-1,5-^{14}C	Control	290
Citrate-1,5-^{14}C	Control	280

TABLE XII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE-Treatment	1,280
Succinate-2,3-^{14}C	PTE-Treatment	1,120
Succinate-2,3-^{14}C	Control	870
Succinate-2,3-^{14}C	Control	910

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE-Treatment	2,540
Succinate-2,3-^{14}C	PTE-Treatment	2,450
Succinate-2,3-^{14}C	Control	1,830
Succinate-2,3-^{14}C	Control	2,140

Experiment 11: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis Prepared from PTE-Treated and Control Animals with Pyruvate-2-^{14}C.

Four thyroparathyroidectomized rats were used in the experiment. Two of the rats received doses of parathyroid extract, and two were injected with vehicle. The rats which received 100 U.S.P. units of parathyroid extract demonstrated an average increase in serum calcium of 2.5 mg% over the level of the control animals.

One-half microcurie of radioactive substrate, 4.0 ml of homogenate and 16.0 micromoles of NADP were present in each flask. The incubation was carried out at 37° C for three hours.

The diaphysis of the femur showed an increase in the oxidation of substrate by the bone homogenate obtained from the PTE-treated animals when compared to that of the control animals. The epiphysis of the femur demonstrated an increase in the oxidation of the radioactive substrate by bone homogenate obtained from the PTE-treated animals (Table XIII).

Experiments 9 - 11 indicate that the addition of NADP is effective in restoring viability to the bone preparations. Femur prepared as NADP fortified homogenates oxidizes citrate-1,5-^{14}C, succinate-2,3-^{14}C and pyruvate-2-^{14}C at a greater rate than control preparations in both diaphysis and epiphysis. In the case of the epiphysis tissue, this is the same pattern of oxidation which was observed using bone fragments (Experiments

TABLE XIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY BONE HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	276
Pyruvate-2-^{14}C	PTE-Treatment	261
Pyruvate-2-^{14}C	Control	202
Pyruvate-2-^{14}C	Control	215

EPIPHYSIS

Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE-Treatment	650
Pyruvate-2-^{14}C	PTE-Treatment	678
Pyruvate-2-^{14}C	Control	383
Pyruvate-2-^{14}C	Control	373

1 - 3). However, the pattern of oxidation in the homogenates of the diaphysis is different from the pattern observed using homogenates of the epiphysis. The rationale for this difference will be discussed in detail in Chapter IV.

It now appears that bone prepared as NADP fortified homogenates from PTE-treated animals will metabolize Krebs cycle and glycolytic intermediates at a greater rate than do control preparation. Concomitant with increased metabolic rates, PTE-treated animals have increased levels of serum calcium. It has yet to be established whether the increased rate of metabolism due to injection of PTE is instrumental in stimulating increased levels of serum calcium or whether elevated levels of serum calcium caused by the injection of PTE are instrumental in increasing rate of metabolism.

In exploring the latter postulate, the following series of experiments were designed to study the in vitro and in vivo effect of calcium ion concentration with no hormonal treatment on bone metabolism.

Experiment 12: The Incubation of NADP Fortified Homogenates of Variable Calcium Ion Concentration Prepared from Femur Diaphysis and Epiphysis of Normal Animals with Pyruvate-2-^{14}C.

Six normal animals were selected for the experiment. The femurs from these animals were prepared in the usual fashion except that the femur diaphysis and epiphysis from the three pairs of animals were homogenized in incubation media containing

6 mg%, 10 mg% and 14 mg% calcium respectively.

One-half microcurie of radioactive substrate, 4.0 ml of homogenate and 16.0 micromoles of NADP were present in each flask. The incubation was carried out at 37° C for three hours.

The magnitude of evolution of $^{14}CO_2$ appeared to parallel the magnitude of calcium ion present in the medium of both homogenates of diaphysis and epiphysis (Table XIV). As the calcium of the medium was increased from 6 mg% to 14 mg%, the total amount of evolved $^{14}CO_2$ was increased.

Experiment 13: The Incubation of NADP Fortified Homogenates Prepared from Femur Diaphysis and Epiphysis of Animals Maintained on Normal and Calcium-deficient Diets with Pyruvate-2-^{14}C.

Of the four thyroparathyroidectomized animals selected for the experiment, two were maintained on a calcium-deficient test diet (Nutritional Biochemical Corp.) and two were maintained on a normal diet for a period of one week. Serum calcium concentrations for the calcium-deficient and normal diet animals averaged 8.5 mg% - 10.6 mg% respectively.

One-half microcurie of radioactive substrate, 4.0 mls. of homogenate and 16.0 micromoles of NADP were present in each flask. The incubation was carried out at 37° C for three hours.

Bone epiphysis and diaphysis prepared from animals maintained on a calcium-deficient diet produced less $^{14}CO_2$ from the substrate than did these bone preparations obtained from animals maintained on a normal diet (Table XV).

TABLE XIV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE AND CONTAINING A VARYING AMOUNT OF CALCIUM ION.

DIAPHYSIS Substrate	Experimental Condition	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	6 mg% Calcium	72
Pyruvate-2-^{14}C	6 mg% Calcium	80
Pyruvate-2-^{14}C	6 mg% Calcium	99
Pyruvate-2-^{14}C	10 mg% Calcium	136
Pyruvate-2-^{14}C	10 mg% Calcium	118
Pyruvate-2-^{14}C	10 mg% Calcium	139
Pyruvate-2-^{14}C	14 mg% Calcium	175
Pyruvate-2-^{14}C	14 mg% Calcium	168
Pyruvate-2-^{14}C	14 mg% Calcium	172
EPIPHYSIS Substrate	**Experimental Condition**	**Activity (cpm/mg bone)**
Pyruvate-2-^{14}C	6 mg% Calcium	207
Pyruvate-2-^{14}C	6 mg% Calcium	206
Pyruvate-2-^{14}C	6 mg% Calcium	218
Pyruvate-2-^{14}C	10 mg% Calcium	283
Pyruvate-2-^{14}C	10 mg% Calcium	295
Pyruvate-2-^{14}C	10 mg% Calcium	310
Pyruvate-2-^{14}C	14 mg% Calcium	354
Pyruvate-2-^{14}C	14 mg% Calcium	360
Pyruvate-2-^{14}C	14 mg% Calcium	347

TABLE XV

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE AND PREPARED FROM ANIMALS ON CALCIUM-DEFICIENT AND NORMAL DIETS

DIAPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	Calcium Deficient	8.8	94
Pyruvate-2-^{14}C	Calcium Deficient	8.3	98
Pyruvate-2-^{14}C	Calcium Deficient	8.4	86
Pyruvate-2-^{14}C	Normal	10.6	120
Pyruvate-2-^{14}C	Normal	10.3	117
Pyruvate-2-^{14}C	Normal	10.9	132

EPIPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	Calcium Deficient	8.8	227
Pyruvate-2-^{14}C	Calcium Deficient	8.3	224
Pyruvate-2-^{14}C	Calcium Deficient	8.4	221
Pyruvate-2-^{14}C	Normal	10.6	391
Pyruvate-2-^{14}C	Normal	10.3	381
Pyruvate-2-^{14}C	Normal	10.9	340

Experiments 12 and 13 indicate *in vitro* and *in vivo* calcium ion concentrations prior to sacrifice can influence bone metabolism *in vitro*. The oxidation pattern presented in the data of Experiment 12 resembles the oxidative pattern presented by the injection of PTE in that both the injection of PTE and high concentrations of *in vitro* calcium ion (14 mg%) stimulate bone oxidation of substrate. Conversely, bone preparation incubated in low concentration of calcium (6 mg%) or prepared from animals maintained on a calcium-deficient diet and having low serum calcium values possessed the least oxidative activity in terms of $^{14}CO_2$ evolution.

To determine whether or not the observed oxidative pattern is consistent in bone prepared from animals made hypocalcemic by injection of TCT, the following section of experiments was designed.

Experiment 14: The Incubation of Fragments Prepared from the Femur Diaphysis and Epiphysis of TCT-Treated and Control Animals with Citrate-1,5-^{14}C.

Four animals were thyroparathyroidectomized and maintained on a normal diet ad libitum for two days after surgery. Then two of the four animals received intraperitoneal injections of 15 mg of thyrocalcitonin TCA powder dissolved in 0.01 N acetic acid in two doses of 0.5 ml over a period of two hours. The control animals received comparable injections of 0.01 N acetic acid. The serum calcium levels of the TCT-treated and control

animals averaged 9.8 mg% and 10.0 mg% respectively.

A weighed quantity (approximately 100 mg) of fragments of femur diaphysis and epiphysis was placed in each flask and to it 4.0 ml of incubation media and one microcurie of radioactive substrate was added. The incubation was carried out at 37° C for 3 hours in a constant temperature shaker water bath.

The labeled carbon dioxide produced from the substrate by bone preparations from the TCT-treated and control animals was not different (Table XVI).

Experiments 15 and 16: The Incubation of NADP Fortified Homogenates of Femur Diaphysis and Epiphysis from TCT-Treated and Control Animals with Citrate-1,5-^{14}C.

Four thyroparathyroidectomized animals were used in these experiments. Seventy-two hours after surgery two of the four animals were injected with 15 mg of thyrocalcitonin TCA powder dissolved in 0.01 N acetic acid in two doses of 0.5 ml each over a two hour period. The control received a comparable volume of 0.01 N acetic acid. Serum calcium values of the TCT-treated and control animals averaged 8.9 mg% and 9.3 mg% in Experiment 15 and 8.6 mg% and 9.0 mg% in Experiment 16.

The femurs were excised, cleaned and homogenized. A 4.0 ml aliquot of homogenate together with one-half microcurie of substrate and 16.0 micromoles of NADP were present in each flask. The incubation was run at 37° C for three hours in a constant temperature water bath.

TABLE XVI

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY FRAGMENTS OF FEMUR DIAPHYSIS AND EPIPHYSIS FROM THYROCALCITONIN-TREATED AND CONTROL ANIMALS.

DIAPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	TCT-Treatment	9.4	41
Citrate-1,5-^{14}C	TCT-Treatment	9.2	38
Citrate-1,5-^{14}C	Control	10.3	44
Citrate-1,5-^{14}C	Control	9.7	36

EPIPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	TCT-Treatment	9.4	140
Citrate-1,5-^{14}C	TCT-Treatment	9.2	135
Citrate-1,5-^{14}C	Control	10.3	150
Citrate-1,5-^{14}C	Control	9.7	131

The results given in Tables XVII and XVIII indicate that TCT-treatment does not affect the evolution of $^{14}CO_2$ from labeled substrate by either diaphysis or epiphysis.

The results of Experiments 14 - 16 indicate that the dose level of TCT administered to the test animals produces a very slight decrease in serum calcium (approximately less than 0.5 mg%) and has no pronounced effect on the oxidation of substrate by bone. This indicates that TCT itself does not affect oxidation of substrates in bone tissue.

Hypercalcemic animals are more sensitive to a given dose level of TCT than are normocalcemic animals. With this in mind, the last series of experiments were designed to produce a greater fall in serum calcium per dose level of TCT which was used previously. The oxidation of substrate by bone was compared in PTE-treated, PTE-TCT-treated and control animals. The dose of TCT which was administered to PTE-treated animals produced approximately a 2 mg% decrease in serum calcium.

Experiments 17, 18 and 19: The Incubation of NADP Fortified Homogenates of Bone Prepared from the Diaphysis and Epiphysis of PTE-Treated, PTE-TCT-Treated and Control Animals with Citrate-1,5-^{14}C.

Six thyroparathyroidectomized animals were used in the experiment. Twenty four hours after surgery, four of the six animals received 100 U.S.P. units each of Injection Parathyroid over a period of two days in two doses of 50 units each. The

TABLE XVII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY BONE HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE AND PREPARED FROM THYROCALCITONIN-TREATED AND CONTROL ANIMALS

DIAPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	TCT-Treatment	8.7	51
Citrate-1,5-^{14}C	TCT-Treatment	8.9	49
Citrate-1,5-^{14}C	TCT-Treatment	9.1	50
Citrate-1,5-^{14}C	Control	9.2	48
Citrate-1,5-^{14}C	Control	9.4	52
Citrate-1,5-^{14}C	Control	9.3	55

EPIPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	TCT-Treatment	8.7	140
Citrate-1,5-^{14}C	TCT-Treatment	8.9	150
Citrate-1,5-^{14}C	TCT-Treatment	9.1	130
Citrate-1,5-^{14}C	Control	9.2	148
Citrate-1,5-^{14}C	Control	9.4	134
Citrate-1,5-^{14}C	Control	9.3	139

TABLE XVIII

LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY BONE HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE AND PREPARED FROM THYROCALCITONIN-TREATED AND CONTROL ANIMALS

DIAPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	TCT-Treatment	8.4	42
Citrate-1,5-^{14}C	TCT-Treatment	8.3	38
Citrate-1,5-^{14}C	TCT-Treatment	9.1	44
Citrate-1,5-^{14}C	Control	8.8	45
Citrate-1,5-^{14}C	Control	8.7	44
Citrate-1,5-^{14}C	Control	9.2	47

EPIPHYSIS

Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	TCT-Treatment	8.4	90
Citrate-1,5-^{14}C	TCT-Treatment	8.3	88
Citrate-1,5-^{14}C	TCT-Treatment	9.1	95
Citrate-1,5-^{14}C	Control	8.8	91
Citrate-1,5-^{14}C	Control	8.7	92
Citrate-1,5-^{14}C	Control	9.2	93

two control animals received no injection whatever. Twelve hours after the last dose of Injection Parathyroid, two of the four animals each received 15 mg of thyrocalcitonin TCA powder dissolved in 0.01 N acetic acid and administered in the form of two injections of one-half ml each over a two hour period. Serum calcium levels prior to sacrifice in the PTE-treated, PTE-TCT-treatment and control animals are indicated in the Tables XIX, XX and XXI.

Bone homogenates were prepared and one-half microcurie of substrate was introduced into each flask as well as 4.0 ml of bone homogenate and sixteen micromoles of NADP. The incubation was carried out at 37° C for three hours in a constant temperature shaker water bath.

The results of the three experiments (Tables XIX, XX and XXI) indicated that the bone diaphysis and epiphysis prepared from PTE-treated animals produces a greater amount of $^{14}CO_2$ from substrate than does bone diaphysis and epiphysis prepared from PTE-TCT-treated animals. Production of $^{14}CO_2$ from substrate in the bone preparation from PTE-TCT-treated animals is greater than that produced from the control animals.

Experiment 20: The Incubation of NADP Fortified Homogenates of Bone Prepared from the Diaphysis and Epiphysis of PTE-Treated, PTE-TCT-Treated and Control Animals with Succinate-2,3-^{14}C.

Six thyroparathyroidectomized animals were used in the experiment. Twenty four hours after surgery, four of the six

TABLE XIX

EFFECT OF PARATHYROID EXTRACT ALONE AND PARATHYROID EXTRACT TOGETHER WITH THYROCALCITONIN IN VIVO ON THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE	13.5	99
Citrate-1,5-^{14}C	PTE	13.2	98
Citrate-1,5-^{14}C	PTE	13.6	103
Citrate-1,5-^{14}C	PTE-TCT	10.7	58
Citrate-1,5-^{14}C	PTE-TCT	11.2	56
Citrate-1,5-^{14}C	PTE-TCT	11.2	55
Citrate-1,5-^{14}C	Control	9.8	20
Citrate-1,5-^{14}C	Control	9.8	19
Citrate-1,5-^{14}C	Control	10.2	19
EPIPHYSIS Substrate	**Experimental Condition**	**Serum Calcium (mg%)**	**Activity (cpm/mg bone)**
Citrate-1,5-^{14}C	PTE	13.5	190
Citrate-1,5-^{14}C	PTE	13.2	182
Citrate-1,5-^{14}C	PTE	13.2	174
Citrate-1,5-^{14}C	PTE-TCT	10.7	119
Citrate-1,5-^{14}C	PTE-TCT	11.2	131
Citrate-1,5-^{14}C	PTE-TCT	11.2	134
Citrate-1,5-^{14}C	Control	9.8	82
Citrate-1,5-^{14}C	Control	9.8	76
Citrate-1,5-^{14}C	Control	10.2	70

TABLE XX

EFFECT OF PARATHYROID EXTRACT ALONE AND PARATHYROID EXTRACT TOGETHER WITH THYROCALCITONIN *IN VIVO* ON THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE	14.0	106
Citrate-1,5-^{14}C	PTE	13.7	99
Citrate-1,5-^{14}C	PTE	13.8	116
Citrate-1,5-^{14}C	PTE-TCT	11.6	91
Citrate-1,5-^{14}C	PTE-TCT	11.4	85
Citrate-1,5-^{14}C	PTE-TCT	11.5	83
Citrate-1,5-^{14}C	Control	9.8	52
Citrate-1,5-^{14}C	Control	9.6	52
Citrate-1,5-^{14}C	Control	9.7	66
EPIPHYSIS Substrate	**Experimental Condition**	**Serum Calcium (mg%)**	**Activity (cpm/mg bone)**
Citrate-1,5-^{14}C	PTE	14.0	404
Citrate-1,5-^{14}C	PTE	13.7	450
Citrate-1,5-^{14}C	PTE	13.8	380
Citrate-1,5-^{14}C	PTE-TCT	11.6	255
Citrate-1,5-^{14}C	PTE-TCT	11.4	262
Citrate-1,5-^{14}C	PTE-TCT	11.5	271
Citrate-1,5-^{14}C	Control	9.8	230
Citrate-1,5-^{14}C	Control	9.6	215
Citrate-1,5-^{14}C	Control	9.7	235

TABLE XXI

EFFECT OF PARATHYROID EXTRACT ALONE AND PARATHYROID EXTRACT TOGETHER WITH THYROCALCITONIN IN VIVO ON THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF CITRATE-1,5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Citrate-1,5-^{14}C	PTE	13.1	274
Citrate-1,5-^{14}C	PTE	12.9	254
Citrate-1,5-^{14}C	PTE	13.2	265
Citrate-1,5-^{14}C	PTE-TCT	11.1	200
Citrate-1,5-^{14}C	PTE-TCT	10.9	192
Citrate-1,5-^{14}C	PTE-TCT	10.8	197
Citrate-1,5-^{14}C	Control	9.2	197
Citrate-1,5-^{14}C	Control	9.4	189
Citrate-1,5-^{14}C	Control	9.1	212
EPIPHYSIS Substrate	**Experimental Condition**	**Serum Calcium (mg%)**	**Activity (cpm/mg bone)**
Citrate-1,5-^{14}C	PTE	13.1	550
Citrate-1,5-^{14}C	PTE	12.9	570
Citrate-1,5-^{14}C	PTE	13.2	565
Citrate-1,5-^{14}C	PTE-TCT	11.1	458
Citrate-1,5-^{14}C	PTE-TCT	10.9	421
Citrate-1,5-^{14}C	PTE-TCT	10.8	436
Citrate-1,5-^{14}C	Control	9.2	420
Citrate-1,5-^{14}C	Control	9.4	470
Citrate-1,5-^{14}C	Control	9.1	439

animals received 100 U.S.P. units each of Injection Parathyroid over a period of two days in two doses of 50 units each. The two control animals received no injections. Twelve hours after the last dose of Injection Parathyroid, two of the four animals each received 15 mg of thyrocalcitonin TCA powder which was dissolved in 0.01 N acetic acid and administered in the form of two injections of one-half ml each over a two hour period. Serum calcium levels prior to sacrifice in the PTE-treated, PTE-TCT-treatment and control animals averaged are indicated in Table XXII.

Bone homogenates were prepared and one-half microcurie of substrate was introduced into each flask as well as 4.0 ml of bone homogenate and sixteen micromoles of NADP. The incubation was carried out at 37° C for three hours in a constant temperature shaker water bath.

As shown in Table XXII the results of the experiment indicate that the bone prepared from PTE-treated animals produces a greater amount of $^{14}CO_2$ from substrate, than does bone prepared from PTE-TCT-treated animals. Production of $^{14}CO_2$ from substrate in bone prepared from PTE-TCT-treated animals is greater than that produced from controls. This pattern is observed in the preparations of both the diaphysis and epiphysis.

TABLE XXII

EFFECT OF PARATHYROID EXTRACT ALONE AND PARATHYROID EXTRACT TOGETHER WITH THYROCALCITONIN *IN VIVO* ON THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF SUCCINATE-2,3-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Succinate-2,3-^{14}C	PTE	12.6	124
Succinate-2,3-^{14}C	PTE	13.1	115
Succinate-2,3-^{14}C	PTE	13.1	126
Succinate-2,3-^{14}C	PTE-TCT	10.4	85
Succinate-2,3-^{14}C	PTE-TCT	10.6	95
Succinate-2,3-^{14}C	PTE-TCT	10.5	80
Succinate-2,3-^{14}C	Control	9.6	70
Succinate-2,3-^{14}C	Control	9.9	75
Succinate-2,3-^{14}C	Control	9.9	85
EPIPHYSIS Substrate	**Experimental Condition**	**Serum Calcium (mg%)**	**Activity (cpm/mg bone)**
Succinate-2,3-^{14}C	PTE	12.6	1,200
Succinate-2,3-^{14}C	PTE	13.1	1,190
Succinate-2,3-^{14}C	PTE	13.1	1,210
Succinate-2,3-^{14}C	PTE-TCT	10.4	910
Succinate-2,3-^{14}C	PTE-TCT	10.6	920
Succinate-2,3-^{14}C	PTE-TCT	10.5	1,010
Succinate-2,3-^{14}C	Control	9.6	925
Succinate-2,3-^{14}C	Control	9.9	950
Succinate-2,3-^{14}C	Control	9.9	960

Experiment 21: The Incubation of NADP Fortified Homogenates of Bone Prepared from the Diaphysis and Epiphysis of PTE-Treated, PTE-TCT-Treated and Control Animals with 2-Oxoglutarate-5-^{14}C.

Six thyroparathyroidectomized animals were used in the experiment. Twenty four hours after surgery, four of the six animals received 100 U.S.P. units each of Injection Parathyroid over a period of two days in two doses of 50 units each. The two control animals received no injections. Twelve hours after the last dose of Injection Parathyroid, two of the four animals each received 15 mg of thyrocalcitonin TCA powder which was administered in the form of two injections of one-half ml each over a two hour period. Serum calcium levels prior to sacrifice in the PTE-treated, PTE-TCT-treatment and control animals averaged 13.1 mg%, 11.6 mg% and 10.1 mg% respectively.

Bone homogenates were prepared and one-half microcurie of substrate was introduced into each flask as well as 4.0 ml of bone homogenate and sixteen micromoles of NADP. The incubation was carried out at 37° C for three hours in a constant temperature shaker water bath.

The results of the experiment shown in Table XXIII indicate that the bone prepared from the PTE-treated animals produces a greater amount of $^{14}CO_2$ from substrate, than does bone prepared from PTE-TCT-treated animals. Production of $^{14}CO_2$ from substrate in bone prepared from PTE-TCT-treated animals is greater than that produced from controls. This pattern is

TABLE XXIII

EFFECT OF PARATHYROID EXTRACT ALONE AND PARATHYROID EXTRACT TOGETHER WITH THYROCALCITONIN IN VIVO ON THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF 2-OXOGLUTARATE-5-^{14}C BY HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS FORTIFIED WITH NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

DIAPHYSIS Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
2-Oxoglutarate-5-^{14}C	PTE	12.9	104
2-Oxoglutarate-5-^{14}C	PTE	13.4	90
2-Oxoglutarate-5-^{14}C	PTE	13.0	96
2-Oxoglutarate-5-^{14}C	PTE-TCT	11.5	61
2-Oxoglutarate-5-^{14}C	PTE-TCT	11.8	57
2-Oxoglutarate-5-^{14}C	PTE-TCT	11.5	55
2-Oxoglutarate-5-^{14}C	Control	10.0	33
2-Oxoglutarate-5-^{14}C	Control	9.8	35
2-Oxoglutarate-5-^{14}C	Control	10.5	32
EPIPHYSIS Substrate	**Experimental Condition**	**Serum Calcium (mg%)**	**Activity (cpm/mg bone)**
2-Oxoglutarate-5-^{14}C	PTE	12.9	420
2-Oxoglutarate-5-^{14}C	PTE	13.4	415
2-Oxoglutarate-5-^{14}C	PTE	13.0	430
2-Oxoglutarate-5-^{14}C	PTE-TCT	11.5	380
2-Oxoglutarate-5-^{14}C	PTE-TCT	11.8	371
2-Oxoglutarate-5-^{14}C	PTE-TCT	11.5	368
2-Oxoglutarate-5-^{14}C	Control	10.0	342
2-Oxoglutarate-5-^{14}C	Control	9.8	386
2-Oxoglutarate-5-^{14}C	Control	10.5	372

observed in the preparations of both the diaphysis and epiphysis.

Experiment 22: The Incubation of NADP Fortified Homogenates of Bone Prepared from the Diaphysis and Epiphysis of PTE-Treated, PTE-TCT-Treated and Control Animals with Pyruvate-2-^{14}C.

Six thyroparathyroidectomized animals were used in the experiment. Twenty four hours after surgery, four of the six animals received 100 U.S.P. units each of Injection Parathyroid over a period of two days in doses of 50 units each. The control animals received no injections. Twelve hours after the last dose of Injection Parathyroid, two of the four animals each received 15 mg of thyrocalcitonin TCA powder which was administered in the form of two injections of one-half ml over a two hour period. Serum calcium levels prior to sacrifice in the PTE-treated, PTE-TCT-treatment and control animals averaged 12.8 mg%, 10.9 mg% and 9.7 mg% respectively.

Bone homogenates were prepared and one-half microcurie of substrate was introduced into each flask as well as 4.0 ml of bone homogenate and sixteen micromoles of NADP. The incubation was carried out at 37° C for three hours in a constant temperature shaker water bath.

The results of the experiment given in Table XXIV indicate that the bone prepared from the PTE-treated animals produces a greater amount of $^{14}CO_2$ from substrate, than does bone prepared from PTE-TCT-treated animals. Production of $^{14}CO_2$ from substrate in bone prepared from PTE-TCT-treated animals is greater than

that produced from controls. This pattern is observed in the preparations of both the diaphysis and epiphysis.

TABLE XXIV

EFFECT OF PARATHYROID EXTRACT ALONE AND PARATHYROID EXTRACT TOGETHER WITH THYROCALCITONIN IN VIVO ON THE LABELED CARBON DIOXIDE PRODUCED FROM THE OXIDATION OF PYRUVATE-2-^{14}C BY LABELED HOMOGENATES OF FEMUR DIAPHYSIS AND EPIPHYSIS

DIAPHYSIS Substrate	Experimental Condition	Serum Calcium (mg%)	Activity (cpm/mg bone)
Pyruvate-2-^{14}C	PTE	12.6	101
Pyruvate-2-^{14}C	PTE	13.0	102
Pyruvate-2-^{14}C	PTE	12.8	98
Pyruvate-2-^{14}C	PTE-TCT	10.7	74
Pyruvate-2-^{14}C	PTE-TCT	11.3	80
Pyruvate-2-^{14}C	PTE-TCT	10.7	79
Pyruvate-2-^{14}C	Control	9.6	23
Pyruvate-2-^{14}C	Control	9.8	21
Pyruvate-2-^{14}C	Control	9.7	25
EPIPHYSIS Substrate	**Experimental Condition**	**Serum Calcium (mg%)**	**Activity (cpm/mg bone)**
Pyruvate-2-^{14}C	PTE	12.6	181
Pyruvate-2-^{14}C	PTE	13.0	190
Pyruvate-2-^{14}C	PTE	12.8	187
Pyruvate-2-^{14}C	PTE-TCT	10.7	159
Pyruvate-2-^{14}C	PTE-TCT	11.3	167
Pyruvate-2-^{14}C	PTE-TCT	10.7	156
Pyruvate-2-^{14}C	Control	9.6	124
Pyruvate-2-^{14}C	Control	9.8	114
Pyruvate-2-^{14}C	Control	9.7	119

CHAPTER IV

DISCUSSION

The work contained in this dissertation was initiated in an attempt to explain the apparent dual metabolic response to parathyroid extract injection which was reported by Cohn (27). He indicated that epiphyseal-metaphyseal femur slices prepared from PTE-treated rabbits metabolized glycolysis intermediates to a greater extent than did comparable control preparations, while similar preparations showed a marked depression in the metabolism of Krebs cycle intermediates. There is no postulated metabolic pathway which could account for this phenomenon.

Yates et al. (117) also presented evidence that PTE does have a dual metabolic role in bone tissue. He varied the levels of endogenous PTH via peritoneal lavage. After sacrifice of the lavaged animal, the femurs were removed and separated into metaphyses (trabecular bone) and diaphysis (compact bone) in preparation for incubation. By measuring citrate and lactate concentrations present in the media, it was demonstrated that citrate production was greater in the diaphysis and lactate production was greater in the metaphysis.

The work indicates that the dual metabolic effect produced by the administration of parathyroid extract might be explained by the cellular differences of compact and trabecular bone. The effects of PTH on citrate production are seen mainly in the diaphysis of the femur. The most numerous cell type of the

diaphysis is the osteocyte although osteoblasts and osteoclasts are also present. The epiphyseal cell types include a mixture of mesenchyme cells, osteoblasts, osteoclasts and osteocytes. In general, the structure of the epiphysis is less highly organized than that of the diaphysis.

Experiments presented by DeLong (35) confirmed the dual response in bone tissue fragments. Representative experiments have been recalculated on the basis of cpm of evolved CO_2/mg bone to facilitate comparison with the experiments described in this dissertation.

TABLE XXV

RESPONSE OF BONE TISSUE FRAGMENTS TO PTE-TREATMENT AS MANIFESTED BY $^{14}CO_2$ EVOLUTION FROM LABELED SUBSTRATE

Substrate	Treatment	Activity Diaphysis cpm/mg bone	Activity Epiphysis cpm/mg bone
Citrate-1,5-^{14}C	PTE	26	21
	Control	50	11
Succinate-2,3-^{14}C	PTE	30	77
	Control	43	54
Pyruvate-2-^{14}C	PTE	373	883
	Control	230	583

In general, the response shown in the metabolism of Krebs cycle acids (citrate and succinate) incubated with bone prepared from PTE-treated animals was that of a depression in $^{14}CO_2$ evolution in the diaphysis and a stimulation in the epiphysis. However, this pattern is not observed during the incubation of pyruvate-2-^{14}C with comparable bone preparations.

Since the structures of compact and trabecular bone are quite different, homogenization was employed to obtain more comparable and uniform preparations. The homogenized bone is not fully viable unless exogenous NADP is added. This finding was also reported by Mecca et al. (78). Results of the incubations of the labeled substrate with NADP fortified homogenates may be summarized as follows:

TABLE XXVI

RESPONSE OF BONE HOMOGENATE TISSUE TO PTE-TREATMENT AS MANIFESTED BY $^{14}CO_2$ EVOLUTION FROM LABELED SUBSTRATE

Substrate	Treatment	Activity Diaphysis cpm/mg bone	Activity Epiphysis cpm/mg bone
Citrate-1,5-^{14}C	PTE	124	375
	Control	56	285
Succinate-2,3-^{14}C	PTE	1200	2495
	Control	890	1985
Pyruvate-2-^{14}C	PTE	268	664
	Control	208	378

The metabolic pattern resulting from a comparison of the oxidation of Krebs cycle acids by NADP fortified bone homogenates prepared from PTE-treated and control animals indicates an increase in $^{14}CO_2$ evolution from labeled substrate in both the diaphysis and epiphysis preparation obtained from the PTE-treated animals. This same pattern is observed during the incubation of pyruvate-2-^{14}C with comparable bone preparation.

The data indicate that the metabolic response of PTE is probably a reflection of the differences in physical structure of trabecular and compact bone rather than a function of cellular distribution or unique metabolic pathways. The diaphysis contains highly organized Haversian system which contains viable cells deep within the mineral matrix. Left intact, it would take longer for isotope to diffuse into the cell in the Haversian system, than such a diffusion in the structurally less organized epiphysis. Diaphysis contains less viable cells on the surface of the mineral than epiphysis. The epiphysis is the chief growing and remodeling center of bone and contains a high concentration of cellular material in the epiphyseal plate area. Therefore diffusion of isotope into the cells contained in compact and trabecular bone probably proceeds at different rates if the tissue structure is left relatively intact which it would be in bone fragment preparations. This variable is eliminated by homogenizing the bone tissue to a small uniform particle size.

Bone tissue can incorporate citric acid as well as other Krebs cycle organic acids into the mineral matrix. This occurs to a greater extent in resorbing areas in the stable calcium compartment. Krebs cycle acids are incorporated into the open spaces in the partially resorbed crystal lattice. The diaphysis is mineralized to a greater extent than the epiphysis in the relatively young animals used in these experiments. Therefore the diaphysis of the PTE-treated animal will tend to bind more tracer acid than will the diaphysis of control animals, since PTE-treated animals have more resorbing sites. This would effectively reduce the amount of available tracer and thus give an erroneously low rate of metabolism in the diaphysis of PTE-treated animals. Homogenization will equalize the number of broken crystal lattice sites in both PTE and control preparations, thus equalizing any substrate binding differences.

Four micromoles/ml of homogenate is the optimal concentration of NADP necessary to obtain a fully viable bone homogenate. At concentrations less than that amount, the system is apparently not sensitive to hormone treatment, or it will exhibit a different metabolic pattern.

Evidence has been presented which indicates that the metabolic changes associated with the administration of PTE are not a direct function of PTH action. Purified PTH has no effect on glycolysis in Erhlich Ascites tumor cells. However, non-hypercalcemic fractions from the gel filtration purification

of PTE does have a metabolic response in this assay similar to that produced by PTE (52).

Aurbach et al. (8) has shown that several metabolic responses previously attributed to PTE action in kidney homogenates, can be duplicated with non-hypercalcemic peptides. In particular, the reported PTH-induced stimulation in ion transport and mitochondrial respiration believed to be involved in the mechanism of action of calcium homeostasis, can be duplicated in a comparable *in vitro* system using non-hypercalcemic fractions obtained during purification of parathyroid hormone as well as basic proteins such as polylysine and prolamine.

The alterations in metabolism produced by the injection of PTE can be measured up to 48 hours after injection. However the half-life of PTH has been estimated to be 20 minutes. Even so, the serum calcium levels remain elevated 24 - 48 hours after a pharmacological dose of PTE.

To study the possible relation between the elevated levels of serum calcium and metabolism, bone homogenates prepared in media containing various concentrations of calcium ion were incubated with pyruvate-2-^{14}C. This substrate was chosen because of its relatively central position in the metabolic pathways of bone. The results of the experiment are summarized in Table XXVII.

TABLE XXVII

RESPONSE OF BONE TISSUE PREPARATION TO IN VITRO CALCIUM LEVELS AS MANIFESTED BY $^{14}CO_2$ EVOLUTION FROM LABELED SUBSTRATE

Substrate	Media Ca^{++} Mg%	Activity Diaphysis cpm/mg bone	Activity Epiphysis cpm/mg bone
Pyruvate-2-^{14}C	14 mg%	162	353
Pyruvate-2-^{14}C	10 mg%	134	294
Pyruvate-2-^{14}C	6 mg%	88	205

As the calcium concentration of the medium was increased from 6 mg% to 14 mg%, the evolution of $^{14}CO_2$ was increased from 88 cpm/mg bone to 162 cpm/mg bone in the diaphysis and 205 cpm/mg to 353 cpm/mg bone in the epiphysis.

In order to assertain whether or not the alterations in metabolism of pyruvate-2-^{14}C produced by varying in vitro calcium ion levels are comparable to the alterations in metabolism produced by varying in vivo serum calcium, bone tissue obtained from thyroparathyroidectomized rats maintained on a normal and a calcium-deficient diet was prepared for incubation (Table XXVIII).

TABLE XXVIII

RESPONSE OF BONE TISSUE PREPARATION TO IN VIVO CALCIUM LEVELS AS MANIFESTED BY $^{14}CO_2$ EVOLUTION FROM LABELED SUBSTRATE

Substrate	Diet	Average Serum Ca^{++}	Activity Diaphysis cpm/mg bone	Activity Epiphysis cpm/mg bone
Pyruvate-2-^{14}C	Normal	10.6 mg%	123	370
Pyruvate-2-^{14}C	Calcium-deficient	8.5 mg%	93	225

The results indicate that the tissue prepared from the animals which have the lower serum calcium values also have a lower rate of bone metabolism. It does appear then, that the levels of calcium can influence the rate of metabolism both in vitro and in vivo

In order to elaborate on this premise, the rate of metabolism in animals made hypercalcemic and hypocalcemic by hormonal treatment was undertaken. The initial goal in this phase of the experimental work was to determine rates of metabolism in normal animals and animals made hypocalcemic by injection of thyrocalcitonin (TCT). The results of these experiments appear in Table XXIX.

TABLE XXIX

RESPONSE OF BONE TISSUE PREPARATION TO TCT-TREATMENT AS MANIFESTED BY $^{14}CO_2$ EVOLUTION FROM LABELED SUBSTRATE

Substrate	Treatment	Average Serum Ca^{++}	Activity Diaphysis cpm/mg bone	Activity Epiphysis cpm/mg bone
Citrate-1,5-^{14}C	Control	9.3	52	140
Citrate-1,5-^{14}C	TCT	8.9	50	140

The data indicates that the slight depression of serum calcium produced by the administration of the particular dose level of TCT has no significant effect on metabolism. Apparently metabolism is sensitive only to large changes in calcium concentration.

Animals are more sensitive to any given dose level of TCT if they are in a state of hypercalcemia. In this regard, a greater sensitivity to TCT can be developed in animals by pretreatment with PTE. The animals made hypercalcemic via PTE administration will show a greater decrease in serum calcium than that decrease in serum calcium produced by the same dose level of TCT injected into an animal with normal serum calcium level.

The series of experiments in the latter portion of the dissertation were designed to study bone metabolism at three different levels of *in vivo* calcium ion concentrations. The results of the experiments appear in Table XXX.

TABLE XXX

RESPONSE OF BONE TISSUE PREPARATION TO PTE AND PTE WITH SUBSEQUENT TCT-TREATMENT AS MANIFESTED BY $^{14}CO_2$ EVOLUTION FROM LABELED SUBSTRATE

Substrate	Treatment	Average Serum Ca^{++}	Activity Diaphysis cpm/mg bone	Activity Epiphysis cpm/mg bone
Citrate-1,5-^{14}C	PTE	13.3 mg%	100	182
	PTE-TCT	11.1 mg%	56	128
	Control	10.0 mg%	19	76
Succinate-2,3-^{14}C	PTE	12.9 mg%	121	1,200
	PTE-TCT	10.5 mg%	86	946
	Control	9.8 mg%	76	945
2-Oxoglutarate-5-^{14}C	PTE	13.1 mg%	96	421
	PTE-TCT	11.6 mg%	57	339
	Control	10.1 mg%	33	366
Pyruvate-2-^{14}C	PTE	12.8 mg%	100	186
	PTE-TCT	10.9 mg%	77	160
	Control	9.7 mg%	23	119

The data indicates that the level of metabolic activity in the bone preparations of diaphysis and epiphysis seems to reflect the level of serum calcium. Hypercalcemia produced by injection of PTE is associated with increased bone metabolic activity compared to the control bone preparations with all substrates tested. Injection of TCT into test animals made hypercalcemic by the administration of PTE appreciably reduced the hypercalcemia. Concomitant with the reduction in serum calcium, a reduction in metabolic activity of the bone preparations was also observed.

CHAPTER V

SUMMARY AND CONCLUSIONS

It has been noted by numerous researchers that the administration of Parathyroid Extract to test animals produces a dual metabolic response in bone. The results of others as well as the data presented in the begining of the Experimental Chapter of this dissertation indicate that the oxidation of glycolytic intermediates is increased during the incubation of surviving femur epiphysis fragments while the oxidation of Krebs cycle intermediates is diminished during the incubation of surviving femur diaphysis fragments. (Table XXV). However, when the bone tissues are homogenized and adequate amounts of NADP are added to reconstitute the system one observes a stimulation in the oxidation of both Krebs cycle and glycolytic intermediate in femur diaphysis and epiphysis prepared from PTE-treated animals (Table XXVI). Homogenization of these tissues minimizes the inherent physical and substrate binding differences which may account for the pattern observed in studies using intact or fragmentized compact and trabecular bone. A consistent pattern of oxidative response to PTE is in itself more logical than the previously reported 'dual' metabolic response, since there are no recognized metabolic pathways which could account for the 'dual' response.

Cohn and Forscher (26) reported that 85% of the total glucose present during incubation with bone was oxidized via

glycolytic - Krebs-cycle pathways while 15% was metabolized via the pentose shunt. Therefore the majority of the labeled CO_2 produced from substrates during bone metabolism is due to Krebs cycle activity.

The metabolic activity of bone homogenate is increased as the level of *in vitro* calcium ion is increased (Table XXVII). Also, bone homogenate prepared from thyroparathyroidectomized animals maintained on a calcium-deficient diet shows a lower metabolic activity than bone homogenates prepared from the thyroparathyroidectomized rats maintained on a normal diet (Table XXVIII). These animals also maintained a lower serum calcium than did animals on a normal diet. It appears that both *in vitro* and *in vivo* levels of calcium ion can influence bone metabolism.

TCT administered to thyroparathyroidectomized rats produces a very slight decrease in serum calcium and has no significant influence on bone metabolism (Table XXIX). However, if the same dose level of TCT is injected into an animal made hypercalcemic by prior administration of PTE, the resultant marked decrease in serum calcium is accompanied by a significant decrease in bone metabolism (Table XXX). The decrease in metabolism must be a direct result of the decrease in serum calcium since the administration of the same dose level to a normal animal produced a slight fall in serum calcium but no significant change in bone metabolism.

The data presented in the dissertation is the first direct evidence of the influence of calcium ion concentration on the oxidation of substrates in bone. It has been demonstrated that PTH does not influence the oxidation of substrates (52). Current evidence indicates that this hormone initiates bone resorption by promoting the release and synthesis of bone destroying enzymes (38). In evaluating the role of bone metabolism in the overall role of bone resorption and accretion, one might speculate that as bone resorption proceeds, intracellular levels of calcium can increase. Increased intracellular levels of calcium during bone resorption can produce, according to the experimental evidence presented herein, an increase in bone metabolism which in turn can produce a localized acidosis at the area of resorption. An acidic medium at the site of resorption will favor resorption, since almost all of the bone destroying enzymes are acid hydrolases. TCT has been shown to promote the accretion of calcium into bone. Calcium accretion will probably involve a decrease in the intracellular levels of calcium. Such a decrease according to the data presented in the dissertation, will reduce the cellular acid products (ie. cellular acidity) and thus diminish the activity of the acid hydrolases.

In conclusion, one can state that bone metabolism is no longer believed to be the primary controlling mechanism of bone resorption and accretion as proposed by Neuman and Neuman (82).

However, it is possible that the rate of bone metabolism as influenced by the intracellular calcium levels can act as a secondary control mechanism which provides an optimal acidic cellular medium for the enzymes of bone resorption. A stimulation in metabolism produced by increased calcium levels during resorption, would favor resorption by causing increased accumulation of the acid products at metabolism thus producing a localized acidosis. The activity of the acid hydrolases involved in resorption would be increased in the acidic medium. This postulate might account for the fact that serum calcium levels remain elevated up to 24 - 30 hours after the injection of PTH even though the half life of the hormone has been shown to be 20 minutes.

Conversely, injection of thyrocalcitonin will arrest bone resorption and the intracellular levels of calcium ion will probably decrease. The decreased oxidative rate due to relatively low intracellular calcium levels would retard the accumulation of the acid products of metabolism and thus retard the activity of bone destroying enzymes.

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APPROVAL SHEET

The dissertation submitted by Allyn F. DeLong has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 27, 1982
Date

Signature of Advisor